Oxidative Repair of a Thymine Dimer in DNA from a Distance by a Covalently Linked Organic Intercalator

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Abstract: A thymine cyclobutane dimer, site-specifically incorporated in a DNA duplex, is shown to be repaired upon photoexcitation (at 380 nm) of a naphthalene diimide intercalator (NDI), either bound noncovalently to the duplex or covalently appended to the C4 amine of a methylated cytosine base well separated from the thymine dimer. The repair of the thymine dimer is triggered by photooxidation either directly or by DNA-mediated charge transport over a distance of ∼22 Å, the separation between NDI and the cyclobutane ring. Photooxidative repair with covalently and noncovalently bound NDI is demonstrated using HPLC under denaturing conditions, where the loss of the thymine dimer-containing strand and the formation of the repaired strand are monitored directly, as well as using a novel gel electrophoretic assay. In this assay, two strands of oligonucleotides containing 5′- and 3′-terminal thymidines are first ligated photochemically to yield thymine dimers, and repair is then assayed by monitoring the reversal of the photoligation by intercalators bound either noncovalently or at a distance. Although both NDI and a rhodium intercalator were seen to reverse the photoligation, several anthraquinones and ethidium were unable to promote repair upon irradiation at 350 nm. This photoligation reversal assay provides a rapid screen for thymine dimer repair. The oxidative repair of thymine dimers in a DNA duplex from a distance appears now to be a general phenomenon and requires consideration in developing mechanisms for DNA-mediated charge transport.

Introduction

The thymine cyclobutane dimer (T<>T) is one of the principal photoproducts formed upon exposure of DNA to UV irradiation. This DNA lesion, if left unrepaired, can be both mutagenic and carcinogenic.[1] It is therefore of interest to understand the mechanisms by which UV-induced photolesions are detected and repaired. In mammalian cells, the thymine dimer lesion, once detected, is removed from DNA by dual incision of the damaged strand on both sides of the lesion, followed by filling of the resulting gap and ligation.[2] Extensive research has been carried out to delineate the mechanism of thymine dimer repair in bacteria, where naturally occurring photolyase enzymes can repair these lesions without excision in a catalytic process initiated by sunlight.[1,3]

The thymine dimer is formed as a result of a [2+2] photocycloaddition reaction between adjacent thymine bases on the same polynucleotide strand (Figure 1). The repair of this lesion can be triggered by electron transfer from the repair protein photolyase. Photolyase initiates the repair by direct reduction of the cyclobutane dimer, and the unstable radical anion, once formed, reverts with oxidation to the repaired form. In model systems, the repair of thymine dimers can be triggered both oxidatively and reductively,[4] since the cyclobutane radical cation is similarly unstable. Recently, it was shown that thymine dimers can also be repaired from a distance in a reaction involving DNA-mediated electron transfer.[5] Upon photoactivation with visible light, a rhodium intercalator was found to

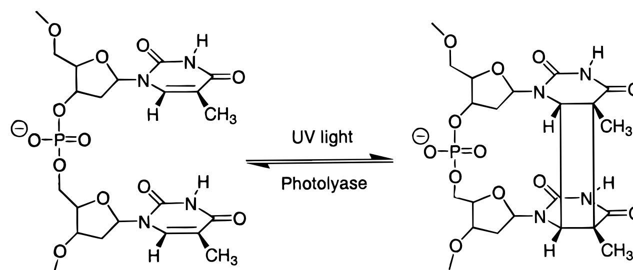

Figure 1. Photoequilibration of a *cis-syn*-thymine dimer.

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trigger oxidatively the repair of a thymine dimer site-specifically incorporated in a DNA oligonucleotide duplex. With non-covalently bound rhodium complex, the reaction is photocatalytic, consistent with electron transfer. Moreover, in assemblies in which the rhodium complex was covalently tethered and intercalated near the end of the duplex, the quantitative repair of the thymine dimer was observed with the photooxidant bound 16 to 36 Å away from the centrally located thymine dimer.

This long-range repair reaction is important to consider in the context of DNA-mediated electron-transfer chemistry. As with other DNA charge transport reactions,[6] the efficiency of thymine dimer repair from a distance was found to be sensitive to perturbations in stacking by the intercalating rhodium complex and also to the integrity of the intervening base stack, but less sensitive to the distance separating the intercalator and thymine dimer. For example, repair was more efficient with the Δ-Rh diastereomer compared to the Λ-Rh isomer, consistent with preferential intercalation of right-handed metal complexes into the right-handed DNA helix,[7] allowing more overlap with the DNA base stack. Furthermore, despite only a small variation in repair efficiency with changes in distance separating the thymine dimer and rhodium, the insertion of base bulges which perturb the base stack caused a marked decrease in the efficiency of repair.

Oxidative damage to DNA from a distance has also been demonstrated in reactions involving DNA-mediated charge transport. The 5′-G of 5′-GG-3′ doublets are sites of low oxidation potential in DNA and are preferentially oxidized.[8] Guanine damage is a more complex reaction than thymine dimer repair, since several steps after the formation of the guanine cation radical by electron transfer are involved.[9] Thymine dimer repair, in contrast, may be viewed simply as a photoisomerization reaction initiated by electron transfer. Many examples of oxidative DNA damage at long range have now been documented,[6,10] while the rhodium intercalator $[Rh(phi)_2(bpy')]^{3+}$, where phi = 9,10-phenanthrenequinone diimine and bpy′ = 4-butyric acid, 4′-methylbipyridine, is the only reported oxidant demonstrated thus far to repair thymine dimers from a distance. This observation may be a consequence of differences in thermodynamics for the two reactions. The oxidation potential of guanine is estimated to be roughly 1.3 V versus NHE,[11] much lower than that for the thymine dimer. The oxidation potential of thymine is estimated to be ~1.7 V vs NHE.[11] It is noteworthy that another metallointercalator, $Ru(phen)(bpy')(dppz)^{3+}$, where dppz = dipyridophenazine, is capable of promoting oxidative DNA damage, but cannot repair thymine dimers.[5b] Based upon the estimated excited-state reduction potentials for $[Rh(phi)_2(dmb)]^{3+}$ (dmb = 4,4′-dimethylbipyridine) $E_{1/2}([Rh]^{3+*/2+})$ = 2.0 V vs NHE[12] and ground-state reduction potentials for $Ru(phen)(bpy')(dppz)^{3+}$ $E_{1/2}([Ru]^{3+/2+})$ = 1.6 V vs NHE,[13] we can bracket the oxidation potential for the thymine dimer in DNA to be between 1.6 and 2.0 V versus NHE.

We have recently found that a naphthalene diimide intercalator (NDI), appended to the C4 amine of a methylated cytosine base, can serve as a long-range DNA photooxidant.[14] NDI and related compounds have been known to display anticancer[15] and antiviral[16] activities that arise from their ability to intercalate into DNA.[17] The photophysical properties of these compounds have also been thoroughly studied,[18] and it has previously been shown that a free naphthalimide chromophore can oxidize guanines via an electron-transfer mechanism.[19] Upon irradiation at 355 nm, photoinduced electron transfer to the lowest electronically excited triplet states of an NDI chromophore from GMP occurs with a rate constant of 2.0×10^7 M^{-1} s^{-1}, considerably faster than that from other nucleotides, although electron transfer was seen in all cases.[18b] The excited-state reduction potential for this NDI chromophore in aqueous solution was estimated to be 1.9 V versus NHE, with a triplet lifetime of 100 μs.[18a] Recent results, however, suggest that electron transfer involving also the excited singlet state may be important. In fact, rapid electron transfer and charge recombination processes initiated by the electronically excited singlet state were found to predominate with DNA-bound NDI.[18a]

Both in the context of developing new synthetic molecules to repair the thymine dimer lesion and in an effort to explore further the parameters governing DNA charge transport chemistry, it was of substantial interest to find another intercalator that, as a potent photooxidant, could trigger the repair of thymine dimers in DNA from a distance. Here we describe assays for thymine dimer repair with several photooxidants (Figure 2) and,

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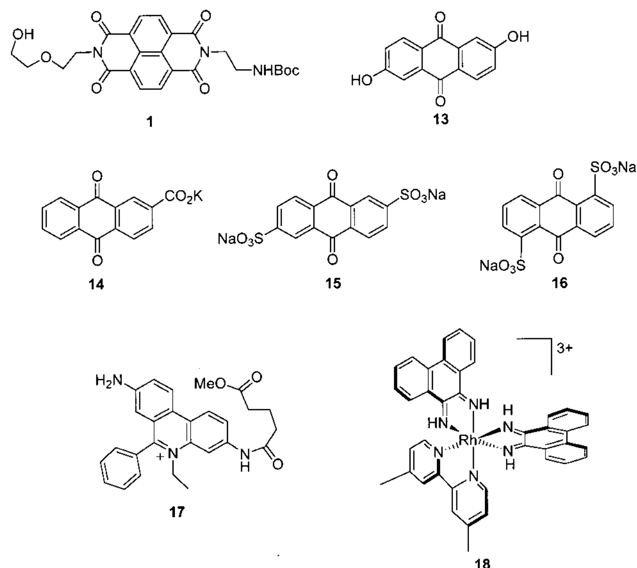

Figure 2. Photooxidants tested to repair thymine dimers in DNA.

in particular, the demonstration of the repair of thymine dimers in DNA at a distance upon photoactivation of NDI.

Experimental Section

Materials and Methods. Oligonucleotides were prepared on an Applied Biosystems 394 DNA synthesizer, using phosphoramidite chemistry.[20] DNA was synthesized with a 5′-dimethoxy trityl (DMT) protective group and was purified by HPLC on a Dynamax 300 Å C_{18} reversed-phase column (10 mm i.d. × 25 cm length) from Rainin on a Hewlett-Packard 1100 HPLC. The DMT group was removed by incubation in 80% acetic acid for 20 min at 20 °C, and then the DNA was HPLC purified again. The concentration of single stranded oligonucleotides in aqueous solution was determined by UV−visible spectroscopy on a Beckman DU 7400 spectrophotometer; ϵ (260 nm, L M^{-1} cm^{-1}), adenine = 15400; guanine = 11500, cytosine = 7400, thymine = 8700, T<>T = 0. Duplexes were formed by cooling solutions containing equimolar quantities of complementary oligonucleotides from 94 to 10 °C over 120 min. $[Rh(phi)_2(bpy')]^{3+}$ and *N*-8-glycyl ethidium were prepared according to previously published procedures.[6,21,22] Thymine dimer formation in synthetic oligonucleotides was performed photochemically, using acetophenone in large excess as a triplet photosensitizer as previously described.[5,23]

Preparations of oligonucleotides with appended naphthalene diimides and rhodium intercalalators have been described elsewhere.[21,24] Appending NDI onto DNA involves functionalizing a thymine nucleotide with an NDI unit, subsequently forming a 5-methylcytosine derivative. The synthesis of all NDI-containing strands was confirmed by MALDI mass spectroscopy, and were all within 2 mass units of the calculated values. Helix stabilization with covalent NDI is evident from melting temperature studies, where duplexes containing tethered NDI melted at least 3 °C higher than those without. Model building indicated that NDI appended onto the base can easily intercalate within the duplex (with a minor roll of the cytosine) and the hydrophobicity of the NDI moiety would favor such intercalative stacking. Some aggregation of the conjugates has been observed over time.

HPLC Assay for Thymine Dimer Repair. Complementary DNA strands were annealed in buffer containing 50 mM sodium chloride, 10 mM sodium phosphate, pH 7.5. Oligonucleotide duplexes (8 μM) were irradiated with a 1000 W Oriel Hg/Xe arc lamp fitted with a monochromator. Reactions run under anaerobic conditions were performed in a resealable vessel where the samples were degassed by 3 freeze−pump−thaw cycles and subsequently flushed with 1 atm of Ar or left under vacuum. Reaction mixtures were analyzed by HPLC at 65 °C on a Sephasil C_{18} reversed-phase column (4.6 mm i.d. × 25 cm length) from Pharmacia, eluting with a gradient of 50 mM $CH_3CO_2NH_4$/MeCN (98:2 to 93:7 over 20 min, isocratic at 93:7 for 10 min, to 50:50 over 5 min, isocratic at 50:50 for 1 min; flow rate = 1.1 mL/min). Under these conditions, the duplexes dissociate into single strands, each of which elutes from the column with a distinct retention time. Oligonucleotides containing a thymine dimer eluted first, followed by the corresponding repaired strand, and then the complement or the naphthaldiimide-containing complement eluted last. Thymine dimer repair was quantitated from peak areas in the chromatograms (normalized for differences in molar absorptivity at the detection wavelength (λ = 260 nm)).

Rapid Screen Photoligation Reversal Assay for T<>T Repair by Noncovalent Intercalators. A ^{32}P-end-labeled and gel-purified oligonucleotide with a 3′-terminal thymidine was added to a mixture of the following: 10 μM 3′-terminal thymidine strand (unlabeled), 10 μM oligonucleotide with a 5′-terminal thymidine, and 10 μM splint strand complementary to their putative ligation product. This mixture was annealed as described above to form a nicked duplex oligonucleotide with a concentration of 10 μM in 25 mM sodium phosphate at pH 7.0. This duplex mixture was then irradiated at 313 nm (4 °C) to generate a T<>T dimer in ~25% of the duplexes. The resultant duplex mixture was used directly in experiments with noncovalent intercalators, and the disappearance of the larger molecular weight material containing the dimer was monitored versus control lanes. Experiments with noncovalent NDI used a 100 μM stock methanolic solution of the intercalator; thus, in experiments using noncovalent NDI, the final solutions also contained ~10% methanol.

Preparation for the Photoligation Reversal Assay for Long-Range T<>T Repair. A mixture of TT and T<>T-containing duplexes was used to screen noncovalent intercalators, as described above. To test covalently tethered intercalators for dimer repair at a distance, the photoligated dimer strand was first gel-purified and was then annealed to the oxidant-containing complementary strand. First the dimer-containing photoligation product was formed as above. The resultant duplex after irradiation was then dried under vacuum, and the photoligation product was separated from the starting material and the complementary strand using denaturing gel electrophoresis. When electrophoresed for protracted periods, the splint strand complementary to the photoligation product containing the thymine dimer runs notably faster than the photoligation product, thus allowing their separation. The photoligated product was isolated by elution at 37 °C into 10 mM Tris-HCl buffer at pH 7.4 and desalted. Oligonucleotides of the same sequence but with continuous phosphodiester backbones and lacking the T<>T lesion were used as carrier in these experiments. Complementary strand containing bound NDI was annealed to carrier and labeled photoligation product. Irradiations were performed as described above at 1 or 10 μM duplex in 25 mM sodium phosphate at pH 7.0. Dimer repair was monitored by denaturing gel electrophoresis as the conversion from the high molecular weight material containing the photoligated dimer to the smaller strands.

Results

HPLC Experiments with Noncovalent NDI. Figure 3 illustrates the DNA duplexes prepared to examine thymine dimer

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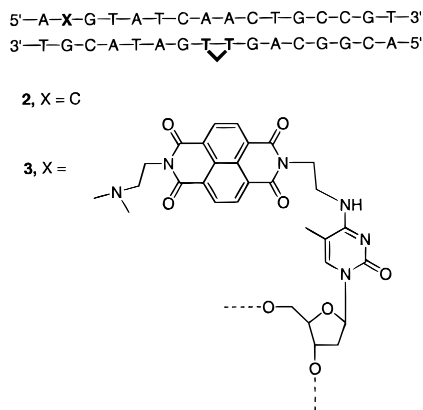

Figure 3. Sequence used to explore thymine dimer repair with NDI bound noncovalently or covalently. Phosphodiester linkages were unmodified.

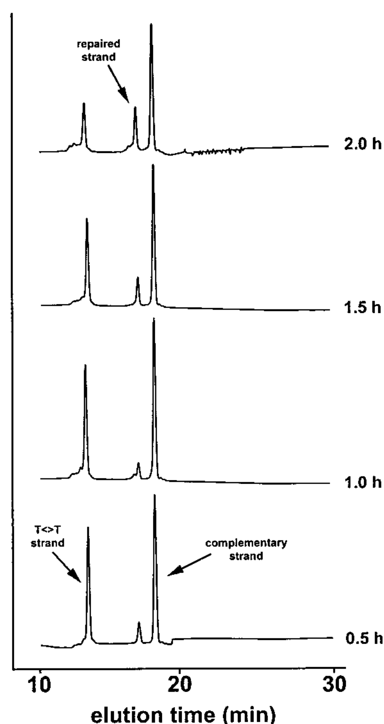

Figure 4. Photochemical repair of a thymine dimer in duplex DNA (8 μM) containing unmodified, phosphodiester linkages by free NDI (8 μM) in sodium phosphate buffer (10 mM) with 50 mM NaCl. Shown are (bottom to top) the HPLC chromatograms at 30, 60, 90, and 120 min of irradiation at 380 nm.

repair in DNA upon photoactivation of NDI. Each duplex contains a thymine dimer site-specifically incorporated in the center of the oligomer as well as a 5′-GG-3′ doublet site. This sequence was used earlier in studies to test the competition between guanine damage and thymine dimer repair by a tethered rhodium intercalator.[5b] In this assay we examine repair by monitoring the conversion of the lesion-containing strand to the repaired strand by HPLC at denaturing temperatures (65 °C) using free or tethered NDI.

As is evident in Figure 4, irradiating duplex **2** under anaerobic conditions with 380 nm light in the presence of free NDI (**1**) results in a substantial amount of dimer repair. We observe both the loss of thymine dimer-containing strand and the formation of the repaired strand. After 2 h of irradiation at 380 nm in the presence of **1** (8 μM), 38% repair of the T<>T containing strand can be seen. No significant damage to the complementary strand could be detected, although minor peaks do appear in the HPLC traces near both parent DNA strands, which we attribute to background oxidative damage under the relatively long irradiations. Interestingly, no repair is evident at low NDI concentrations (<2 μM).

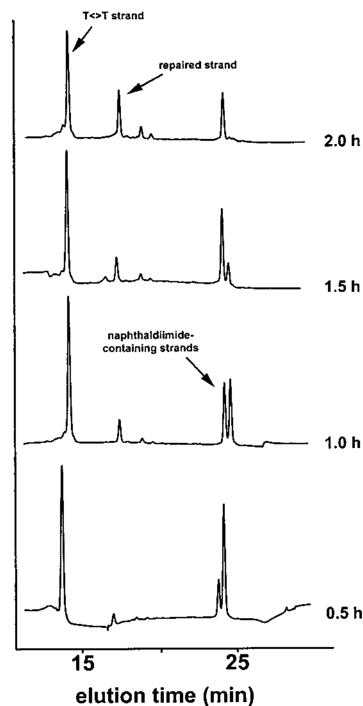

Figure 5. Photochemical repair of a thymine dimer in DNA containing unmodified, phosphodiester linkages by tethered NDI (8 μM duplex) in sodium phosphate buffer (10 mM) with 50 mM NaCl. In the DNA assembly, covalently attached NDI is separated from the thymine dimer by ~22 Å. Shown are (bottom to top) the HPLC chromatograms at 30, 60, 90, and 120 min of irradiation at 380 nm.

HPLC Experiments with Covalent NDI. Irradiating duplex **3**, which contains a covalently incorporated NDI spatially well-separated from the thymine dimer, at 380 nm under anaerobic conditions also results in a significant repair of the thymine dimer (Figure 5). After 2 h, 25% of the thymine dimer-containing strand was repaired, similar to the extent of repair seen for noncovalent NDI. It should also be noted that the duplex contains a 5′-GG-3′ site, and we observed in gel experiments that oxidation of the 5′-G in the 5′GG-3′ site does take place under aerobic conditions (data not shown), consistent with the fact that charge transfer is occurring from the tethered NDI group.[14] Under anaerobic conditions, the oxidation of guanine is suppressed, thus simplifying the HPLC analyses.

The HPLC traces also reveal that during this repair process, the NDI-functionalized strand converts to a new species. The new, naphthaldiimide-containing strand was found to be 14 mass units less than the NDI-containing parent strand, as measured by MALDI-TOF mass spectrometry. A similar mass loss was seen in experiments by Saito and co-workers, who found that irradiating a thymine derivative in the presence of a nitro-substituted 1,8-naphthalimide chromophore led to a demethylated product that arose from hydrogen atom abstraction from the thymine methyl group.[25] Given that the 5-methylcytosine is in close proximity to the naphthaldiimide chromophore, such a demethylation reaction in our system seems quite feasible. The persistence of the absorbance at 380 and 365 nm after

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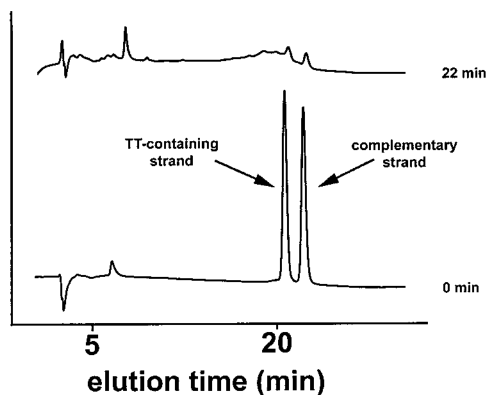

Figure 6. Anthraquinone-2,6-disulfonate (8 μM) mediated decomposition of DNA (8 μM duplex; 5′-ACGTATCAACTGCTGT-3′). Shown are the HPLC analyses before (bottom) and after (top) irradiation for 22 min at 350 nm in 10 mM sodium phosphate, pH 7.6, 50 mM NaCl under aerobic conditions.

irradiation suggests that the NDI chromophore remains intact. Additionally, given the high molecular weight of the DNA strand, it can be concluded that the chromophore is still attached to DNA. Conversion of the NDI-containing parent strand to the new strand is also evident upon irradiation of the NDI-containing parent strand in the absence of its complement.[26] It is noteworthy that repair of the thymine dimer continues even after complete conversion to the new naphthaldiimide-containing strand. Also it should be noted that the relative amount of NDI-containing strand observed in the HPLC traces was found to decrease somewhat during the course of irradiations (Figure 5). We attribute this result either to precipitation or to some retention of the NDI-strand on the C_{18} column.

We also compared these results to those obtained upon irradiation of DNA in the presence of an anthraquinone at much shorter irradiation times. Anthraquinones are also potent photooxidants which have been utilized in studies of DNA charge transport.[10] As evident in Figure 6, and in sharp contrast to our observations with NDI, efficient decomposition of DNA by free anthraquinones appears to be the predominating reaction when the irradiations were monitored by HPLC. It is noteworthy that these conditions are comparable to those used in previous studies and that anthraquinones require oxygen for efficent charge separation in DNA.[10] Given the HPLC analyses, we were very concerned about any application of anthraquinones as sensitizers for oxidative thymine dimer repair in DNA.

A critical issue to establish was whether the NDI-mediated repair occurs only in an intraduplex fashion. This was a concern, as a small amount of decomposition of the NDI strand could be detected with long time irradiations (Figure 5, retention time ~17 min, mass = 4854.3). To test this question, a control reaction under the same conditions and total concentration of reagents was conducted in which the tethered naphthaldiimide-containing strand was annealed to the native strand, and subsequently mixed together with an equal amount of duplex containing the thymine dimer but lacking bound NDI (Figure 7). Upon irradiation at 380 nm for 2 h under anaerobic conditions, only 2% repair was measured, confirming the intraduplex nature of the reaction described above. This control reaction demonstrates that a diffusible species cannot be responsible for repair of the thymine dimer. Therefore, while a small level of interduplex repair can be measured, it is certainly

(26) In separate experiments where an NDI chromophore was end-linked to the 5′-hydroxyl group of an adenine base of an oligonucleotide, the irradiations did not lead to the loss of 14 mass units (unpublished results).

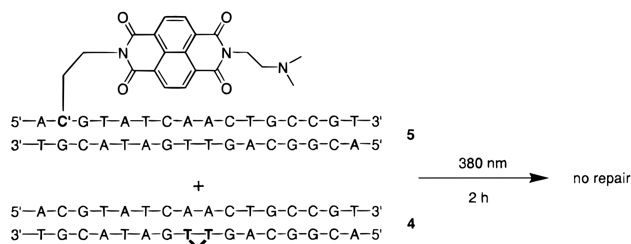

Figure 7. Control experiment demonstrating the intraduplex nature of the repair process. Approximately 2% repair was evident in this control compared to 25% repair for the intraduplex reaction. To achieve repair, then, the NDI and thymine dimer must be in the same duplex.

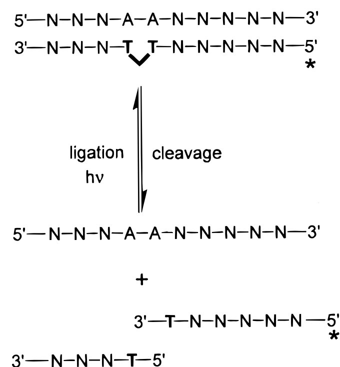

Figure 8. Schematic diagram showing the template-directed photoligation of oligonucleotides effected by cyclobutane dimer formation. An asterisk denotes the position of the radioactive label. Since there is no phosphate linkage between the thymine dimer, ligated versus cleaved strands can be readily assayed by gel electrophoresis.

insufficient to account for the greater than 10-fold higher level of repair seen with covalently bound NDI.

Hence, these data establish that, with photoactivation, NDI can promote the repair of a thymine dimer incorporated in a DNA duplex from a remote site. Here the distance separating NDI and the thymine dimer (taken as the distance between an intercalated NDI and the center of the cyclobutane ring and assuming 3.4 Å stacking between bases) is ~22 Å.

Photoligation Reversal Assay for Thymine Dimer Repair. We have also developed an additional, convenient assay to test for thymine dimer repair by tethered and nontethered intercalators using gel electrophoresis. It has been shown that DNA can act as a template to ligate photochemically two strands of oligonucleotides containing 5′- and 3′-terminal thymidines (Figure 8).[27] Note that the resultant thymine dimer lacks a phosphodiester linkage. The proportions of different possible thymine dimer isomers obtained by photoligation have not been established. If two strands of DNA are held together by a bridging thymine dimer without a phosphodiester linkage, then one can assay thymine dimer repair simply by monitoring the conversion of the high molecular weight strand to the "repaired" and therefore smaller molecular weight species using denaturing gel electrophoresis. Figure 9 shows the duplexes prepared to test for repair. This assay provides a rapid and general screen for thymine dimer repair activity of candidate molecules.

The first set of experiments employed a 41-mer duplex with the photoligated strands consisting of one 18-mer and one 23-mer, and the complementary strand consisting of 41 bases (duplex **6,** Figure 9). The 18-mer was radioactively end-labeled, mixed with the unlabeled 23-mer, and then annealed to the 41-mer complement. After irradiating this nicked duplex for 1 h at

(27) (a) Lewis, R. J.; Hanawalt, P. C. *Nature* **1982**, *298*, 393−396. (b) Liu, J.; Taylor, J.-S. *Nucleic Acids Res.* **1998**, *26*, 3300−3304.

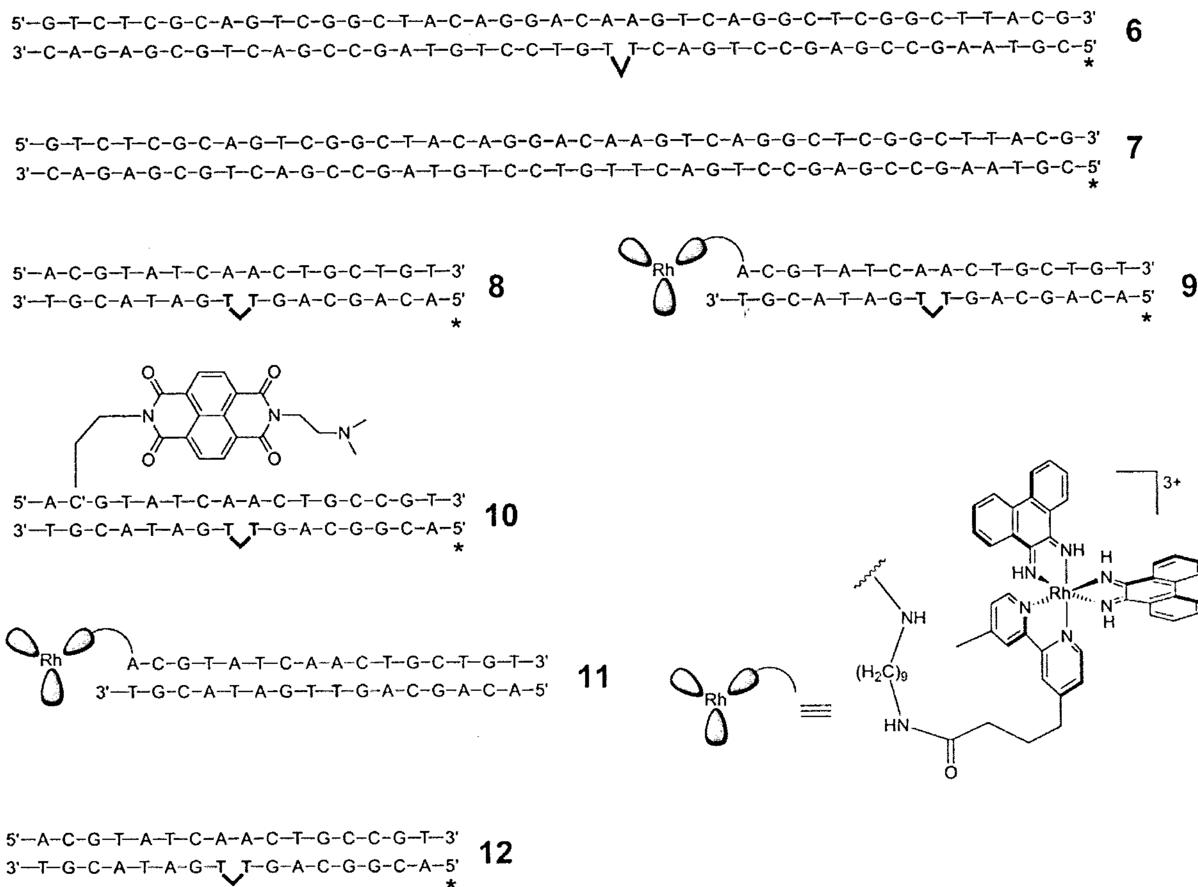

Figure 9. Sequences of the duplexes used to monitor the repair of photoligated thymine dimers. **10** and **12** are identical in sequence to duplexes **3** and **4**, respectively, except for the absence of a phosphodiester linkage between the thymine dimer.

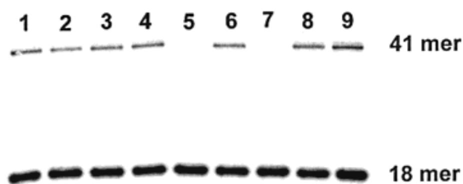

Figure 10. Autoradiogram of the denaturing gel illustrating the reversal of the thymine dimer in duplex **6**, which does not contain a phosphodiester linkage between the thymine dimer. All experiments were done under aerobic conditions, with DNA (10 μM) and intercalators (10 μM) in sodium phosphate buffer (25 mM). The top band represents the 41-mer while the bottom band represents the 18 mer. The loss of the 41-mer band is indicative of repair. Lanes 1−6, irradiation at 350 nm for 30 min in the presence of anthraflavic acid (**13**), anthraquinone-2-carboxylate (**14**), 2,6-disulfate anthraquinone (**15**), 1,5-disulfate anthraquinone (**16**), NDI (**1**), and *N*-8-glycyl ethidium (**17**). Lane 7, irradiation for 30 min at 365 nm in the presence of $[Rh(phi)_2(dmb)]^{3+}$ (**18**). Lane 8, irradiation at 313 for 1 h to form T⟨ ⟩T ligation, then irradiation for 30 min at 350 nm. Lane 9, irradiation at 313 nm for 1 h.

313 nm, photoligation of the 18-mer and 23-mer to form a new 41-mer is evident. The photoligation can be monitored by denaturing gel electrophoresis, since the labeled, photoligated 41-mer runs more slowly than the parent 18-mer (Figure 10). For this assay, the 41-mer duplex product was not subsequently separated from the reactants used for the photoligation. Hence, signal from background radiolabeled 18-mer is evident in all lanes. Nonetheless, the extent of repair can be quantitated by measuring changes in the ratio of 41-mer to 18-mer. With this sample containing the photoligated duplex **6**, we then screened a variety of intercalating compounds to test for thymine dimer repair activity.

Four different anthraquinones were tested for their ability to repair thymine dimers, since anthraquinones have previously been shown to oxidize guanines from a distance in double stranded DNA.[10] Anthraflavic acid (**13**), anthraquinone-2-carboxylate (**14**), 2,6-disulfate anthraquinone (**15**), and 1,5-disulfate anthraquinone (**16**) (see Figure 2) were all tested, but as shown in Figure 10, no repair of the thymine dimer could be detected with these anthraquinones after 30 min of irradiation at 350 nm. To verify that the anthraquinones were bound to DNA under these conditions, a separate experiment was performed where **14** was added to the native 41-mer duplex **7**, irradiated for up to 60 min at 350 nm, and piperidine-treated to reveal DNA damage. Under these conditions, we observed that the anthraquinone oxidized the 5′-G of 5′-GG-3′ sites, consistent with an electron-transfer reaction (data not shown). Thus, although the anthraquinone was able to bind to and oxidize guanine in the 41-mer duplex **7**, it was unable to repair the thymine dimer duplex in **6**. It is noteworthy that the anthraquinones used in this study are very similar in composition to anthraquinone-2-sulfonate, a complex that was used to split thymine dimers in model systems.[28]

Figure 10 also reveals the results of irradiating duplex **6** with noncovalent NDI, *N*-8-glycyl ethidium (**17**), and $[Rh(phi)_2(dmb)]^{3+}$ (**18**). As is shown on the gel, only NDI and $[Rh(phi)_2(dmb)]^{3+}$ were capable of repairing the photoligation, as indicated by the loss of the 41-mer band. Upon repair of the thymine dimer, the labeled 18-mer is released and travels much faster in the denaturing gel. No significant repair was observed with *N*-8-glycyl ethidium upon irradiation at 350 nm. At 313

(28) Young, T.; Nieman, R.; Rose, S. *Photochem. Photobiol.* **1990**, *52*, 661−668.

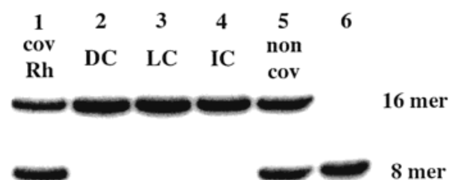

Figure 11. Autoradiogram of the denaturing gel showing reversal of a thymine dimer with free and tethered $[Rh(phi)_2(bpy')]^{3+}$. All experiments were done under aerobic conditions, with DNA that does not contain a phosphate linkage between the thymine dimer (1 μM) and intercalator (1 μM) in sodium phosphate buffer (25 mM). Repair is assayed by monitoring cleavage to form the 8-mer. Lanes 1 and 2, irradiation of duplex **9** at 365 nm for 1 and 0 h, respectively. Lane 3, irradiation of duplex 10 for 1 h. Lane 4, interduplex control, irradiation of duplexes **8** and **11** for 1 h. Lane 5, irradiation of duplex **8** with an equimolar amount of $[Rh(phi)_2(dmb)]^{3+}$. Lane 6, dark control containing radiolabeled 8-mer.

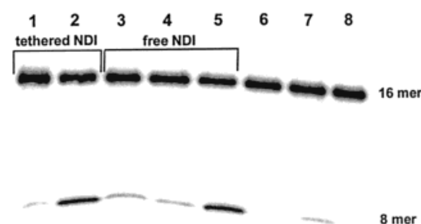

Figure 12. Autoradiogram of the denaturing gel showing reversal of a thymine dimer with free and tethered NDI. All experiments were done under anaerobic conditions, with DNA containing a thymine dimer lacking the interresidue phosphate linkage (10 μM) and intercalator (10 μM) in sodium phosphate buffer (25 mM). Repair is assayed by monitoring cleavage to form the 8-mer. Lanes 1 and 2, irradiation of duplex **10** at 380 nm for 0 and 2 h, respectively. Lanes 3−5, irradiation of duplex **12** at 380 nm for 0 h, 0 h in the presence of free NDI, and 2 h in the presence of free NDI. Lane 6, interduplex control (mixture of duplexes **5** and **12**) after 2 h of irradiation at 380 nm. Lane 7, duplex **12** irradiated in the absence of intercalator. Lane 8, irradiation of anthraquinone-2-carboxylate and duplex **12** for 2 h at 350 nm.

nm, where photolysis of bound ethidium promotes the oxidation of guanine, dimer repair could not be distinguished from photoequilibration of the dimer. This assay is a simple screen, and may provide a means to test rapidly other compounds for thymine dimer repair activity.[29]

Long Distance Repair Assayed by Photoligation Reversal. We were interested in testing thymine dimer repair with covalently bound oxidants using the photoligation reversal assay as well as in examining the repair of DNA duplexes that were similar in composition to those used in the HPLC experiments. To prepare duplexes **8−10**, an 8-mer was radiolabeled and gel purified, and then mixed together with the second 8-mer and annealed to the appropriate 16-mer complement that did not contain functionalized chromophore. The photoligation was effected for 2 h at 313 nm (4 °C), and then the radiolabeled, ligated 16-mer was gel purified a second time under denaturing conditions. Thus the photoligated products were isolated from the 8-mer starting material and the 16-mer splint. The labeled, photoligated 16-mer was then annealed to the appropriate complements giving duplexes **8−10**.

Long-range thymine dimer repair initiated by $[Rh(phi)_2(bpy')]^{3+}$ was tested using the photoligation reversal assay. Figure 11 shows the result of irradiating duplexes **8** (with free rhodium complex **18**) and **9** for 1 h at 365 nm. Repair for covalently bound rhodium was 66%, while that for noncovalently bound rhodium was 44%. An interduplex control reaction (lane 4, Figure 11) revealed only 2% repair, demonstrating that a diffusible species in the reaction mixture cannot account for the observed repair. It is noteworthy that the ratio of repair seen between covalent and noncovalent rhodium in the photoligation reversal assay is much greater than that seen previously,[5] and may be a consequence of the structural and electronic properties of the DNA sequence employed. The photoligation experiment with rhodium involves a somewhat different base sequence, but also a thymine dimer, or a mixture of thymine dimer isomers, that do not contain a phosphodiester linkage. These structural properties may serve to insert the thymine dimer deeper into the base stack and thus render it more accessible to migrating charge.

We examined dimer repair upon anaerobic irradiation of duplexes **10** and **12**, which contain covalent and noncovalent NDI, respectively (Figure 12). Substantial repair occurred after 2 h in both cases (27% for both covalent and noncovalent NDI) and is readily apparent, since under the denaturing conditions of the gel, the radiolabeled "8-mer" migrates much faster than the ligated "16-mer". Duplex **5** was irradiated in the presence of an equimolar amount of duplex **12** for 2 h at 380 nm as a control to test for any interduplex repair. Only 1% of interduplex repair could be detected by gel electrophoresis. The results, shown in Figure 12, demonstrate that the repair due to a diffusible species produced throughout the course of the reaction or from any interduplex interactions with the tethered naphthaldiimide cannot account for the reaction of the covalently bound assembly. Here too, under anaerobic conditions, no repair could be observed by irradiating duplex **12** in the presence of free anthraquinone-2-carboxylate for 2 h at 350 nm.

(29) To minimize possible degradation by those oxidants that are efficient sensitizers of singlet oxygen, reactions may be run anaerobically.

Discussion

Here we have demonstrated that a thymine dimer in DNA can be repaired by long-range electron transfer to NDI with photoactivation. The repair is triggered from an oxidant located ∼22 Å from the lesion, using duplex DNA as a bridge for charge transport. A rapid screening assay for thymine dimer repair was additionally developed. Using this assay, noncovalently bound NDI as well as the rhodium intercalator were demonstrated to repair thymine dimers in DNA, where under the same conditions a variety of anthraquinones could not.

Given that both the phi complex of rhodium[5] and the organic NDI intercalator have now been shown to promote oxidative repair of thymine dimers, it is interesting to compare their reactivity. HPLC experiments reveal that the efficiency of repair by the noncovalent NDI oxidant appears to be somewhat lower than that found for the rhodium complex, and gel assays on the thymine dimers lacking a phosphodiester linkage show the repair efficiencies to be roughly comparable. Perhaps more noteworthy is the comparison between repair from a distance versus noncovalently with both oxidants. Earlier we observed that the repair efficiency with noncovalently bound metal complex was ∼30 times greater than that observed from long range.[5] Here the sequence differs slightly from that studied earlier, but in the case of covalently bound rhodium, we are now monitoring the repair of the mixture of thymine dimers lacking phosphodiester linkages. We find for the rhodium intercalator, as with NDI, that repair from a distance or with noncovalently bound oxidant is comparable. In the case of the gel assays, possibly the conformation of these presumably more flexible thymine dimers permits better overlap within the base pair stack. For NDI, however, equivalent efficiencies using covalent and noncovalent oxidants were seen also in the HPLC assays using true thymine dimers with the phosphodiester linkage. It may

then also be the case that, compared to the metallointercalator, where the tether on the metal complex may somewhat restrict optimal overlap of the intercalating unit with the base stack, the NDI-based intercalators have greater surface area available for stacking; certainly they are extremely hydrophobic.

It is also important to consider those photooxidants for which no repair is observed, either from a distance or directly when noncovalently bound. We earlier saw that for $[Ru(phen)(bpy')(dppz)]^{3+}$, although efficient guanine oxidation by DNA-mediated charge transport can be observed, no thymine dimer repair is evident. We ascribed that result to the reduction potential of the Ru^{3+} species, estimated to be 1.6 V vs NHE and thus insufficient to oxidize the dimer. In the case of NDI, which can promote thymine dimer repair, the reduction potential is at least 1.9 V versus NHE. Our results with anthraquinones, however, suggest that redox potential may not be the sole determinant for predicting candidate molecules for thymine dimer repair. Anthraquinone-2,6-disulfonate (**15**) is reported to have an excited-state triplet reduction potential of 2.4 V versus NHE in aqueous solution,[30] yet repair with noncovalent anthraquinones could not be detected under our experimental conditions.

In addition to achieving the appropriate thermodynamics, the excited-state character of the oxidant may also have to be tailored to suit the electronic properties of the substrate. Thymine dimer formation and splitting via a concerted mechanism are photochemically allowed via an excited singlet state.[31] There are many examples that suggest thymine dimerization/splitting reactions result from an excited singlet state.[32,33] In fact, the naturally occurring enzyme photolyase repairs thymine dimers by electron transfer from the singlet excited state of $FADH_2$ in high quantum yields.[34] It should be noted that oxidative reactions of NDI bound to DNA have recently been proposed to proceed from the excited singlet state.[18a] In contrast, triplet energy transfer to thymine dimers in DNA is known to be an inefficient process.[32] This could be one reason we were unable to detect repair using anthraquinones, molecules whose intersystem crossing to the triplet state is extremely efficient, particularly within DNA.[35] It is noteworthy that anthraquinones have been shown to repair thymine dimers in model systems, albeit with irradiation in the UV range, but not in DNA. Moreover, splitting of thymine dimers by triplet sensitizers in model systems usually proceeds in low quantum yields due to geminate recombination of the monomer radical pair.[36] Our results caution against relating repair in a DNA duplex to these models. In fact, Schuster has questioned studies of rhodium repair from a distance based upon the poor repair efficiency of anthraquinones.[37] It should, however, be self-evident that repair from a distance within a DNA duplex cannot be achieved if the noncovalently bound oxidant is unable to promote repair in DNA.

Given that the oxidant has the necessary characteristics to repair thymine dimers in DNA, oxidative repair of thymine dimers from a distance appears now to be a more general phenomenon and not specific to rhodium intercalators. How then does the charge migrate from one end of the DNA duplex to the thymine dimer located in the middle of the duplex? The fact that thymine dimer repair can occur from a remote site implies that charge is not localized exclusively on guanines, the base of lowest oxidation potential. It has previously been suggested that long-range charge transfer occurs via a hopping mechanism in which charge tunnels between DNA bases of low redox potentials.[10f,g] Here, the thymine dimer is of significantly higher oxidation potential than the guanine, and yet repair is observed. Hence, some charge, however transient, must reside on the thymine dimer. A mechanism that involves a hole hopping from base to base from an oxidant capable of oxidizing all the bases in DNA can account for our long-range repair results, as could a mechanism that invokes a delocalized band model. Previous work has demonstrated that the NDI chromophore does have the appropriate redox potential to oxidize all of the bases in DNA in aqueous solution.[18a] Alternatively, the mechanism of thymine dimer repair may require a combination of hopping and delocalized band formation, and such mechanistic models are starting to emerge.[10b−c,38] It is also interesting that a model has appeared which explains preferential thymine dimer formation in long pyrimidine tracts by invoking delocalization of adsorbed singlet energy through the π-stack in DNA.[31a] Given these results, it will be important also to understand the fundamental principles governing this chemistry in the context of other DNA-mediated electron-transfer reactions. Measurements of rates and efficiencies of repair with different oxidants, and as a function of intervening DNA sequence and structure, will be critical in delineating mechanisms of DNA charge transport.

Finally, our results raise the intriguing possibility that DNA-targeted therapeutics can be designed to function from a distance, using DNA as a bridge for charge transport. This would constitute a new approach to drug design, since most DNA-targeted therapeutics rely on site-specific interactions between the drug and DNA to carry out a specific transformation.[39] Moreover, electron transfer reactions need not be directed only to the thermodynamic "sink" within the duplex. Indeed, in the case of thymine dimer repair, the reaction must be kinetically, rather than thermodynamically, limited. In the systems examined thus far, however, it is the case that guanine oxidation may occur along with repair of the dimer. This poses limitations for the utility of rhodium and NDI-based photochemistry for the therapeutic repair of thymine dimers. However, our results with anthraquinones also suggest that one may be able to tune the acceptor molecule to carry out a *specific* reaction in DNA (i.e. guanine oxidation versus thymine dimer repair). In this regard, a better understanding of the salient features of DNA-mediated electron-transfer chemistry may make it possible to develop redox active chemotherapeutic agents that function from a distance. Certainly, the photoligation assay developed in this study may prove to be helpful in finding such molecules.

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