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Catalytic DNAs That Harness Violet Light To Repair Thymine Dimers in a DNA Substrate

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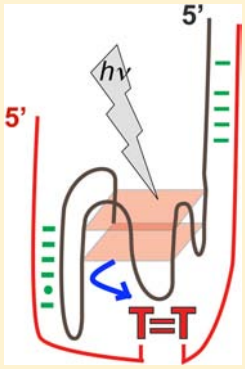
Supporting Information

ABSTRACT: UV1C is an *in vitro* selected catalytic DNA that shows efficient photolyase activity, using light of <310 nm wavelength to photo-reactivate CPD thymine dimers within a substrate DNA. We show here that a minimal mutational strategy of substituting a guanine analogue, 6MI, for single guanine residues within UV1C extends the DNAzyme's activity into the violet region of the spectrum. These 6MI point mutant DNAzymes fall into three distinct functional classes, which photo-reactivate the thymine dimer along different pathways. Cumulatively, they reveal the *modus operandi* of the original UV1C DNAzyme to be a surprisingly versatile one. The interchangeable properties of no less than six of the G→6MI point mutants highlight UV1C's built-in functional flexibility, which may serve as a starting point for the creation of efficient, visible light-harnessing, photolyase DNAzymes for either the prophylaxis or therapy of UV damage to human skin.



INTRODUCTION

Thymine dimers are the most significant lesions formed in cellular DNA from exposure to solar ultraviolet B (290−320 nm) light. Two major classes of thymine dimers are most commonly formed in mammalian cells—the cyclobutane (CPD) and the less abundant but more mutagenic 6-4 dimers (reviewed in refs 1 and 2). Unrepaired, these lesions pose a formidable challenge to cellular DNA replication; indeed, defects in cellular thymine dimer repair machinery have been linked both with human skin cancers and such diseases as Xeroderma pigmentosum.[1,2] For this reason, organisms have evolved diverse strategies for repairing thymine dimers and pyrimidine dimers in general. The most direct approach, for either the CPD or 6-4 product lesions, is to use light in the ultraviolet A-to-visible range (320−450 nm) to directly repair (photo-reactivate) the dimers back to thymines.[1,2] Though photolyases are found in all kingdoms of life, mammals have lost the genes coding for photolyases.[1,2] Nevertheless, it has recently been shown that mice transgenic for *Arabidopsis* CPD photolyase show "superior resistance to sunlight-induced tumorigenesis".[3] Another remarkable study reports that the topical application of *A. nidulans* photolyase-containing liposomes to human skin, followed by exposure to damaging UV light, leads to a significant level of repair of CPD thymine dimers in the DNA of epidermal cells.[4]

The functional architecture of a typical CPD photolyase (protein) enzyme is schematized in Figure 1a. Two classes of light-absorbing cofactors augment the function of these proteins: first, an antenna pigment (variously, a folate derivative, MTHF; or deazaflavin) that harvests 350−450 nm wavelength light, and transfers this excitation energy, non-radiatively, to the second cofactor, a reduced flavin ($FADH^-$). The photoexcited $FADH^-$ then transfers an electron to a thymine dimer recipient that has been flipped out of a distorted DNA duplex (thymine dimers stack poorly with their neighboring bases and also base pair poorly with complementary adenines, leading to their relatively facile mobility in and out of the double helix[5]). The resulting CPD thymine dimer radical anion now reverts rapidly to thymine bases.[1,2]

The discovery of catalytic RNAs, or ribozymes, in the 1980s, encouraged the formulation of the RNA World hypothesis,[6−8] which posits that prior to the evolution of RNA−DNA−protein-based life forms, RNA-containing cells constituted a primitive life, in which RNA served both as information carrier and catalyst. We have been interested to know, for the purpose of defining the range and limits of RNA catalysis, whether catalytic RNAs (ribozymes) or their DNA surrogates (deoxyribozymes or DNAzymes) are capable of catalyzing *photochemical* reactions, specifically, biochemical reactions that require light. In 2004 we initiated an *in vitro* selection experiment (SELEX),[9] to see if we could select from a random-sequence library of single-stranded DNAs ($\sim 10^{14}$ sequences) catalysts capable of harnessing UV-A light (>300 nm wavelength, and at the time thought to be less damaging for DNA, although some evidence to the contrary has since been reported[10]) to photo-reactivate CPD thymine dimers within a specified DNA substrate.[9] Our selection strategy was inspired by the mechanism of proteinaceous bacterial photolyases, as well as that of a catalytic antibody reported for the photochemical repair of thymine dimers.[11,12] In the latter a tryptophan residue, positioned close to the bound thymine

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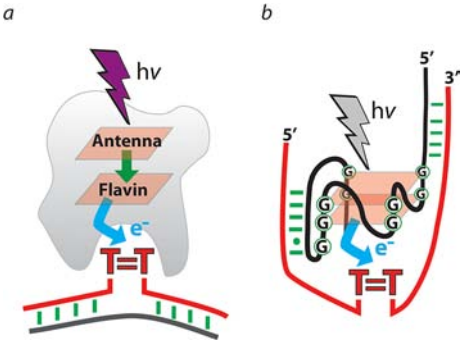

Figure 1. Schematic illustrations of the key functional components of a proteinaceous photolyase enzyme versus a photolyase DNAzyme. (a) A proteinaceous photolyase enzyme (shown in gray). Light ($h\nu$) is absorbed by both the flavin and antenna (MTHF or deazaflavin) chromophores. The photoexcited antenna chromophore transmits its energy by Förster resonance energy transfer to the flavin cofactor. The excited flavin then transfers an electron to a thymine dimer extruded out of a DNA duplex into the photolyase's active site. Functionally, the flavin cofactor alone is sufficient for photo-reactivation. However, the antenna pigment substantially enhances the light-harvesting capability and hence the efficiency of photo-reactivation by the photolyase. (b) The UV1C DNAzyme (shown in black), bound to its single-stranded DNA substrate, TDP (shown in red). It is hypothesized that guanines within a G-quadruplex formed within UV1C absorb light in the 300−310 nm wavelength range, followed by transfer of an electron by one or more photoexcited guanines to the thymine dimer located within the TDP substrate.

dimer substrate, was sufficient for the lesion's photoreactivation[12]

For our *in vitro* selection we created a special DNA substrate that incorporated a *cis,syn*-cyclobutane thymine dimer but lacked the linking phosphodiester between the dimer thymines.[9] . Photo-reactivation of such a substrate necessarily resulted in two shorter pieces of DNA, which enabled us to purify the catalytic sequences away from the larger pool. Serotonin was included in our selection as a flavin substitute.[9] Unexpectedly, two quite distinct thymine-dimer reactivating DNAzymes were cloned from this *in vitro* selection: "Sero1C", a DNAzyme that required serotonin for activity,[5,13] and "UV1C", which catalyzed photo-reactivation without the aid of any extraneous cofactor.[9]

Sero1C, requiring serotonin for activity, had an action spectrum that stretched to ∼340 nm;[13] by contrast, UV1C's action spectrum extended only to ∼315 nm, with the most optimal activity (defined as $k_{catalyzed}/k_{uncatalyzed}$) occurring at 305 nm[9,13]. UV1C had a requirement for sodium ions for catalytic activity (its folded structure incorporated a G-quadruplex,[16] specifically stabilized by Na^+).[9] UV1C was capable of multiple-turnover catalysis, and had a catalytic efficiency comparable to those of catalytic antibodies reported for the same activity.[9,11,12] The only energetically reasonable catalytic mechanism possible for UV1C (Figure 1, *b*) was that its guanine-quadruplex (G-quadruplex[14]) functioned both as light antenna (G-quadruplexes have a higher absorbance in the 300−310 nm wavelength range than DNA duplexes[15]) as well as electron source for thymine dimer photo-reactivation[9] Quantum yield measurements made in the 250−320 nm range showed a

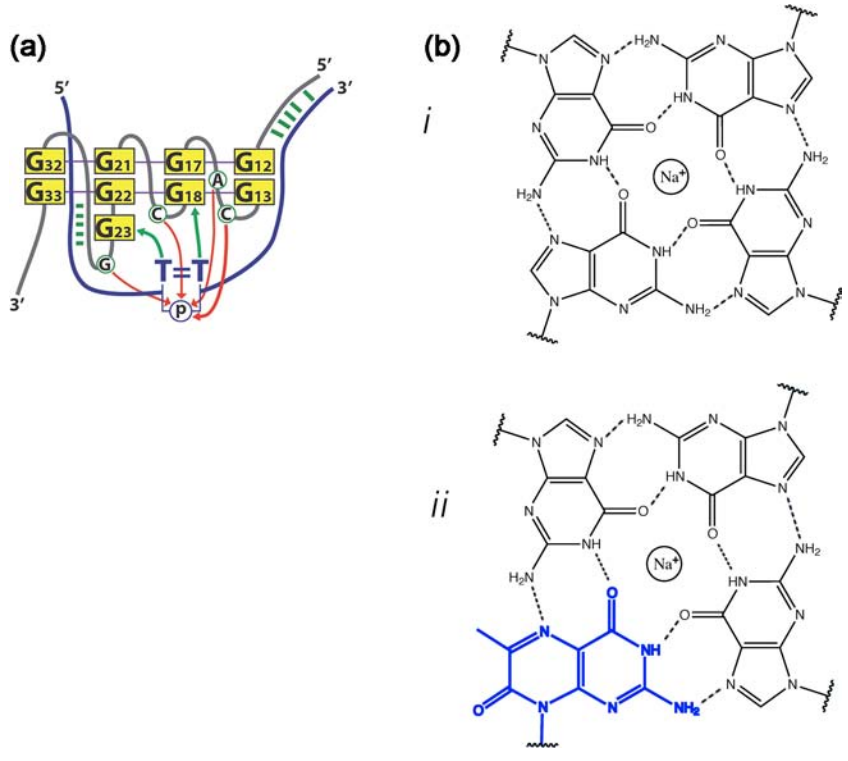

Figure 2. Guanine quadruplex within the UV1C-TDP complex, and a guanine quartet incorporating a 6MI residue. (a) The UV1C DNAzyme, complexed to the TDP substrate, folds to an intramolecular, wholly parallel-stranded G-quadruplex. Red and green arrows indicate the loci of contact cross-links between the thymine dimer (or a phosphorothioate residue placed between the two thymidines forming the dimer) and various nucleobases of UV1C.[15,16] Sites of the different G→6MI point mutations in UV1C are indicated by yellow squares. (b) A guanine quartet (i) and a base-quartet composed of three guanines and one 6MI residue (ii).

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maximal value of 0.05 at 305 nm. While this number is substantially lower than the quantum yields typical of CPD photolyases,[1,2] it matches those of other naturally occurring photolyases (such as those that repair 6-4 thymine dimers[1,2]).

We have recently made strides in understanding the structure−function relationships of UV1C, and these add credence to of our mechanistic hypothesis for this DNAzyme.[9,13,16] First, methylation protection experiments showed that eight specific guanines within UV1C (guanines 12, 13, 17, 18, 21, 22, 32, and 33; Figure 2a) were involved in forming the G-quadruplex. G23, though part of a contiguous GGG stretch, was not a participant in the G-quadruplex. Second, two distinct kinds of contact cross-linking experiments[16,17] confirmed the close spatial proximity (required for efficient electron transfer) between the substrate's thymine dimer and the DNAzyme's G-quadruplex (the key contact cross-links formed between the thymine dimer and DNAzyme bases are summarized in Figure 2a). Based on the above, and mutational studies, we were able to refine our structural/topological model for UVIC folded and complexed with its DNA substrate, TDP (Figure 2a), which incorporated the thymine dimer to be repaired.[9]

The action spectrum of UV1C extends up to ∼315 nm; however, naturally occurring proteinaceous photolyases typically utilize light well into the visible spectrum (<450 nm[1,2]). A DNAzyme that hypothetically finds utility in either the prophylaxis or therapeutics of UV damage to human skin, would benefit from the ability to harness light in the 400−450 nm range, because at such wavelengths there is little danger of new thymine dimer formation in DNA. We therefore considered if it might be possible to modify the structure and base composition of UV1C in minimal ways, so as to endow it with the ability to photo-reactivate CPD thymine dimers with UV-A and/or visible light.

A significant proportion of proteinaceous photolyases utilize a folate/pterin cofactor, MTHF, as their antenna pigment.[1,2] The chemical structure of MTHF reveals a striking similarity between its pterin ring and the nucleobase guanine. On this basis, we explored the utility of a commercially available fluorophore, 6-methylisoxanthopterin (6MI),[18,19] which shares the pterin ring of MTHF and the complete set of hydrogen-bonding sites of guanine. Indeed, 6MI has been shown to be able to replace one or more guanine within G-quartets (Figure 2b), with relatively little structural perturbation.[20] Mergny and co-workers found that the rate of formation of a parallel, intermolecular quadruplex from 5′-TGGMGGT (where M stands for 6MI) actually exceeds that of quadruplex formation by the unmodified DNA, 5′-TGGGGGT. Additionally, the stability of the resultant 6MI-containing quadruplex is only modestly lower than that of the quadruplex formed by 5′-TGGGGGT.[20] Most compelling of all, 6MI, absorbs strongly in the near-UV to visible region of the spectrum, with an absorption maximum at ∼345 nm.

We generated nine G→6MI point mutants of the "wild-type" UVIC DNAzyme (WT UV1C), with each mutant incorporating a single 6MI into the G-quadruplex, or replacing G23 (which does not participate in the quadruplex yet contact cross-links strongly to the substrate's thymine dimer). We wished to examine the catalytic as well as light usage properties of these mutants, relative to those of the original WT UV1C DNAzyme. The nine individual mutation sites are shown in yellow in Figure 2a.

RESULTS AND DISCUSSION

Three Functional Classes of UV1C G→6MI Point Mutants. We carried out two different sets of experiment to evaluate the action spectra and catalytic properties of the nine G→6MI mutant DNAzymes. First, to obtain a broad overview of the properties of the mutant DNAzymes, they were complexed with the TDP substrate and irradiated at nine different wavelengths in the 305−400 nm range from a tunable laser (Supporting Information Figure S-1 shows the experimental setup). Irradiation at each wavelength was carried out at a constant power output of 10 mW (under these conditions repair rates varied linearly with laser power), for an invariant duration of 10 min each. All experiments were carried out under single turnover conditions, with the DNAzyme being present at three orders or magnitude higher concentration than the TDP substrate. Unmutated UV1C ("WT UV1C") was used as a positive control, and LDP, a single-stranded DNA oligomer incapable of folding to a G-quadruplex, as a negative control. As described above, photo-reactivation of the TDP substrate leads to the formation of two smaller DNA strands, and the time-dependent formation of these strands was monitored using denaturing gel electrophoresis (Figure S-2). The results (% TDP photo-reactivated following 10 min of irradiation at individual wavelengths in the 305−400 nm range) are shown in Figure 3. The positive control, WT UV1C, was expected to be

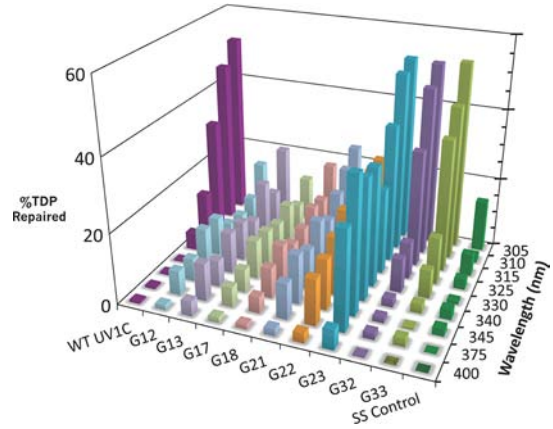

Figure 3. Light usage profiles of the G→6MI point mutants of UV1C. Bar graphs showing the percentage of substrate (TDP) repaired as a function of irradiation with light (wavelengths in the 305−400 nm range), at an invariant flux of 10 mW, for 10 min each.

active in the 305−315 nm range, at the red edge of DNA's absorption spectrum. Indeed, by 325−330 nm, UV1C's catalytic activity is very low, indistinguishable from that of the negative control ("SS DNA").

The behavior of the six G→6MI mutant DNAzymes falls into three distinct categories. First, the mutants G32 and G33 (which incorporate their G→6MI mutations at the G32 and G33 sites, respectively) show action spectra and photo-reactivation rates notably similar to those of WT UV1C. These mutants are catalytically inactive in the 340−400 nm wavelength range, where 6MI absorbs, suggesting that in these DNAzymes the photo-excited 6MI residues cannot serve as effective electron sources for TDP repair, either for steric or other reasons. A second category of DNAzymes, comprising six mutants (G12, G13, G17, G18, G21, and G22), shows a surprisingly homogeneous behavior of enabling a low level of photo-reactivation across the 305−400 nm spectral range. The

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most interesting behavior, however, is shown by the G23 DNAzyme, which constitutes a functional category of its own. It shows a high level of TDP repair across the 305−400 nm wavelength range.

The above experiments, though broadly informative, yielded only a "percent TDP repaired" figure for each DNAzyme; such a figure did not necessarily correlate with a rate constant (such as k_{obs}), because it was uncertain whether in a given experiment we sampled a linear response range, or were close to activity saturation. To obtain authentic k_{obs} values from linear initial rates of photo-reactivation, we tested the activity of all nine DNAzyme mutants at two wavelengths: 305 nm (the optimal functional wavelength for WT UV1C) and 345 nm (the peak of 6MI's absorption spectrum). The k_{obs} values obtained (from two sets of independent experiments) are listed in Table 1. At

Table 1. Observed photo-reactivation rate constants (k_{obs}), at 305 and 345 nm, for the TDP substrate in the presence of excess unmutated UV1C DNAzyme (WT UV1C), or different UV1C G→6MI point mutant DNAzymes

	k_{obs} (h^{-1})	
	at 305 nm	at 345 nm
Double-stranded control	1.8 ± 0.7	0.3 ± 0.1
Single-stranded control	2.1 ± 0.7	0.3 ± 0.1
WT UV1C	230 ± 30	0.4 ± 0.4
G12	2.7 ± 0.7	3.2 ± 0.7
G13	3.0 ± 0.7	5.0 ± 0.7
G17	3.0 ± 0.7	4.0 ± 0.7
G18	2.6 ± 0.7	4.0 ± 0.7
G21	4.0 ± 0.7	6.1 ± 0.7
G22	3.9 ± 0.7	4.0 ± 0.4
G23	344 ± 1.1	19.4 ± 0.4
G32	310 ± 25	0.4 ± 0.4
G33	370 ± 10	0.7 ± 0.4

both 305 and 345 nm wavelength, the existence of TDP as either single-stranded oligomer or component of a duplex, makes little difference to its low (background) rate of photo-reactivation. However, when TDP is complexed with WT UV1C, a >100-fold enhancement of repair occurs at 305 nm, while at 345 nm it remains at the background level.

The G32 and G33 mutants show a photo-reactivation profile similar to that of WT UV1C (Figure 3). It is likely that at 305 nm these guanines (32 and 33) do not participate in thymine dimer repair, possibly because they do not lie sufficiently close to the thymine dimer to serve as efficient electron donors (indeed, earlier cross-linking studies suggested precisely such a lack of proximity[16,17]).

The six mutants DNAzymes of the second functional class—all mutated within their G-quadruplexes (*vide infra*)—are surprisingly poor at photo-reactivation at 305 nm. However, at 345 nm they are consistently 10−20-fold more effective at photo-reactivation than either of the negative controls, WT UV1C as well as the G32 and G33 DNAzymes. The comparable behavior of all six mutants in this class is consistent with the functional redundancy of these six quadruplex guanines. In particular, it suggests that *no single* guanine within WT UV1C or the mutant DNAzymes may have the role of sole electron supplier to the thymine dimer (Figure 4).

How then to account for the poor photo-reactivation by these six DNAzymes at 305 nm? One possibility is that a G→6MI substitution at any of these six positions either abolishes or

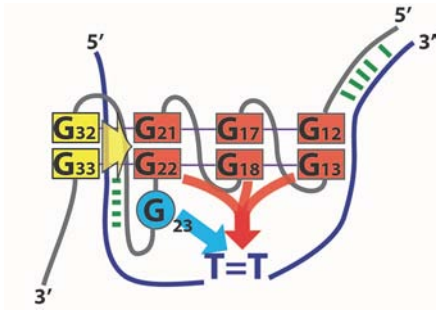

Figure 4. Diagram summarizing likely paths of charge and energy transfer within the DNAzyme−substrate complex. The nine G→6MI point mutant DNAzymes are divided into three functional categories. G32 and G33 are DNAzymes whose action spectrum resembles that of the unmodified (WT) UV1C DNAzyme. DNAzymes G12, G13, G17, G18, G21, and G22 show similar photo-reactivation behavior: they photoreactivate poorly at 305 nm, but more strongly than WT UV1C at >305 nm. DNAzyme G23 is located outside the G-quadruplex, and photo-reactivates at high rates in the entire 305−400 nm range. Blue and red arrows show alternative electronic pathways to the substrate's thymine dimer. The yellow arrow indicates a putative quadruplex stabilization.

destabilizes the DNAzyme's G-quadruplex. To test this, we carried out irradiation experiments at 345 nm with WT UV1C and the G13, G17, and G23 DNAzymes (G13 and G17 belonging to this second functional class, above), and found that all the above DNAzymes photo-reactivated the substrate at 345 nm with far higher rates in the standard, sodium-containing, irradiation buffer (20 mM Tris, pH 7.4, 2 mM EDTA, and 200 mM NaCl), compared to in a buffer where all Na^+ had been replaced with Li^+ (Figure S-3). This result clearly indicates that G-quadruplexes do indeed form within *all* the above DNAzymes (Na^+ stabilizes G-quadruplexes, while Li^+ does not). Could it be that such quadruplexes differ structurally from, or fold with lower efficiency than, the catalytically optimal G-quadruplex formed within WT UV1C? To test these ideas, we first measured the CD spectra of WT UV1C, bound to LDP, as well as of the nine mutant DNAzymes (all bound to LDP). Figure S-4 displays these data cumulatively, juxtaposed with the CD spectra of WT UV1C bound to its complementary oligonucleotide (to give a B-DNA duplex); as well as the spectrum of a standard, parallel-stranded G-quadruplex,[14] $(5'\text{-}T_7G_5A)_4$. The parallel-stranded G-quadruplex standard shows a characteristic positive peak at 265 nm,[21] while the B-DNA duplex shows a characteristic peak at 280 nm.[21] All 10 DNAzyme-LDP spectra show a broad positive peak in the 260−290 nm region; this peak is likely a composite of contributions from the predicted parallel-stranded G-quadruplex within each DNAzyme along with those from duplex elements within the DNAzyme-LDP complexes. Figure S-5 shows that, interestingly, the amplitudes of the 260−290 nm positive peak from the different DNAzyme-LDP complexes (shown are the spectra of WT UV1C, G12, G21, and G33) vary significantly (high for WT and G33; lower for G12 and G21) relative to the almost invariant duplex signals generated from all four DNAzymes. This may reflect the relative levels of G-quadruplex formed in the four DNAzyme−LDP complexes.

An additional detrimental factor for the catalytic properties of six mutants DNAzymes of the second functional class may be the formation of charge transfer complexes between their 6MI residues and neighboring, stacking guanines (resulting in $6MI^{\bullet-}$ and $G^{\bullet+}$).[22] The UV−vis spectra attributable to the presence of

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6MI in different DNAzyme-substrate complexes (Figure S-6) do indeed show modest, but definite, shifts in the absorption spectra, which is consistent with the above notion.[23]

In contrast to the mutant DNAzymes of the second class, the unique and efficient photo-reactivation profile of DNAzyme G23, shown in Figure 3, is borne out by its k_{obs} numbers (Table 1): at 305 nm its k_{obs} values are, respectively, 1.5-fold higher than that of WT UV1C and ~170-fold higher than those of the negative controls; at 345 nm, they are ~70-fold higher than those of either negative control or WT UV1C. The G23 G→6MI mutant, therefore, efficiently photo-reactivates the substrate thymine dimer throughout the λ = 305−400 nm range. As described above, cross-linking experiments on WT UV1C complexed with TDP analogues had established that the G23 residue lies in close proximity to the thymine dimer. However, the G23 residue has also been shown to be non-essential for the 305 nm photo-reactivation by WT UV1C, since it can be mutated to inosine (which has a higher ground state oxidation potential than guanine—1.5 V compared to 1.29 V, both relative to NHE[15]) without any discernible reduction in the DNAzyme's catalytic function.[15,16] How then does the G23 mutant DNAzyme achieve such a strong photo-reactivation function? Two possibilities, not mutually exclusive: (1) electron transfer from 6MI at the G23 position occurs via direct contact with the thymine dimer (distinct from the likely more complex electronic path to the thymine dimer from 6MIs located in the *distal* of the two quartets of the quadruplex); and (2) being extra-helical, the photoexcited 6MI residue at G23 likely does not form significant charge separation complexes with proximal guanines. If hypothesis (2) is true, the fluorescent lifetime of the G23 DNAzyme should exceed that of the G22 DNAzyme, for instance. Future experiments will investigate this question.

Is Efficient Photoreactivation by the G23 G→6MI DNAzyme Wholly a "Proximity" Effect? Given the strong catalytic performance of the G23 mutant DNAzyme across the 305−400 nm wavelength range, we asked whether this capability arose exclusively from the close spatial proximity of its 6MI and the thymine dimer. If this was so, could the observed properties of the G23 DNAzyme be replicated in a structurally unrelated system, where a 6MI residue was deliberately positioned close to a thymine dimer? To investigate this possibility we constructed two test duplexes, both incorporating the TDP substrate as a component strand. The complementary strand in these duplexes was either one or other of the DNA oligomers, X and Y:

X: 5′-ACTCGTACGCACAC(6MI)TACATGTAG-3′

Y: 5′-ACTCGTACGCACAC(6MI)ATACATGTAG-3′

The oligomer X offers a perfect complement to TDP, except for a single 6MI residue ("M" in Figure 5a) substituting for the "AA" sequence that would normally base pair to TDP's thymine dimer. This duplex, DS1, might be expected to have a kinked structure, such as shown in Figure 5a. The oligomer Y incorporates an additional adenine, offering the sequence "6MI A" across from TDP's thymine dimer (in duplex DS2). In both the DS1 and DS2 duplexes, the 6MI residue and the thymine dimer are positioned proximal to each other. Figure 5b plots the k_{obs} values, measured at 305 and 345 nm, of (i) a complex of TDP and the G23 DNAzyme; (ii) DS1; and (iii) DS2. It is evident that photo-reactivation k_{obs} values of all three TDP-containing complexes at 345 nm are close, adding credence to the notion that photo-reactivation by the G23 DNAzyme at this

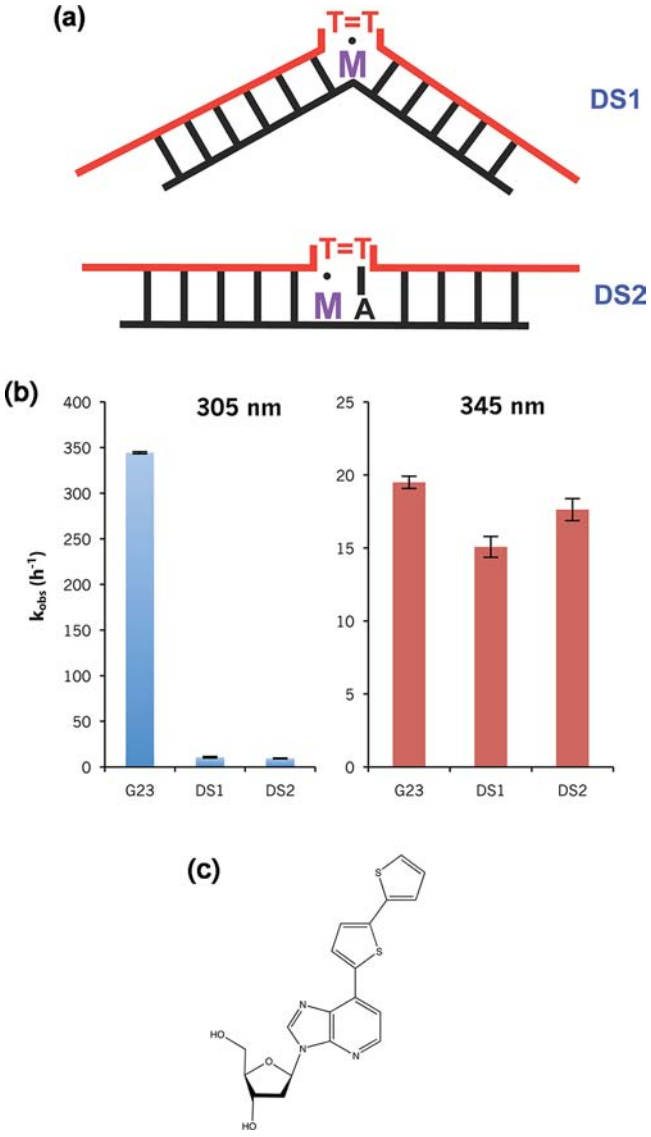

Figure 5. Testing the role of the G23 site within the UV1C DNAzyme. (a) Two test duplexes, DS1 and DS3, in each of which TDP is a constituent strand. The duplexes are designed to position a 6MI residue in close proximity to TDP's thymine dimer. (b) Photo-reactivation k_{obs} values, at 305 and 345 nm, for a TDP-G23 DNAzyme complex ("G23") and the DS1 and DS2 duplexes. (c) Chemical structure of a DSS {7-(2,2′-bithien-5-yl)-imidazo[4,5-*b*]pyridine} nucleotide.

wavelength results from the proximity of its 6MI chromophore and the thymine dimer. However, the 305 nm k_{obs} values for the three TDP complexes are strikingly different: the G23 DNAzyme photo-reactivates the thymine dimer 2 orders of magnitude faster than either DS1 or DS2. Clearly, at 305 nm, 6MI's proximity to the thymine dimer is not a determining factor. Most likely, in the G23 DNAzyme, electron transfer for photo-reactivation at 305 nm occurs from a source distinct from the 6MI, i.e., one or more of the quadruplex guanines. These data help reinforce the notion that both the WT and mutant UV1C DNAzymes have multiple, likely redundant, options for transferring photo-reactivating electrons to the thymine dimer.

A Variety of Chromophores Can Functionally Substitute for Guanine at the G23 Position of UV1C. Given

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our understanding, above, that (a) guanine 23 of WT UV1C 6MI is not a participant in the DNAzyme's G-quadruplex and (b) 6MI placed at the 23 position can efficiently photoreactivate at 345 nm, we reasoned it may be possible to place divergent chromophores at this locus of the DNAzyme, and so obtain UV1C variants capable of utilizing visible light more effectively. We therefore substituted a structurally and spectroscopically distinct dye nucleoside at position 23: 7-(2,2′-bithien-5-yl)-imidazo[4,5-b]pyridine, or DSS[24] (Figure 5c). The absorption spectrum of the G23→DSS mutant DNAzyme is shown in Figure S-7. This G23→DSS mutant DNAzyme, complexed with TDP, was irradiated at 400 nm or at 420 nm. Following 30 min of irradiation at 400 nm, a G13→6MI control DNAzyme showed ∼10% TDP repair; a G23→6MI control DNAzyme showed ∼15% repair. However the G23→DSS DNAzyme showed ∼43% repair. At 420 nm (where 6MI no longer absorbs), the two control DNAzymes had zero activity; however, the G23→DSS DNAzyme showed 27% repair. These experiments confirmed that it was indeed possible to position a variety of chromophores at the G23 site of UV1C, and thereby gain significant flexibility over the desired action spectrum of the resulting DNAzyme.

CONCLUSIONS

The data reported here cumulatively show that WT UV1C, as well as a number of UV1C mutants that incorporate base-analogue dyes such as 6MI and DSS, represent an outstanding and adaptable catalytic system, able to carry out a biocatalytic function *in vitro* comparable to that carried out by more complex proteinaceous photolyase enzymes *in vivo*. Figure 4 summarizes our current understanding of the multiple ways that UV1C and its variants are able to photo-reactivate the TDP thymine dimer.

Regarding the precise mechanism of thymine dimer photoreactivation in these mutant DNAzymes, it is in principle possible that the photoexcited 6MI moiety acts as a photooxidant rather than a photoreductant, repairing the thymine dimer *via* the formation of the latter's radical cation rather than an anion (both the radical cation and anion are highly unstable and revert rapidly to thymine bases[25]). However, it is difficult at this point to carry out a reliable Rehm−Weller analysis[26] on the likely direction of charge flow, given lack of information on such key parameters as the oxidation potential of the CPD thymine dimer, as well as the oxidation and reduction potentials of photoexcited 6MI nucleotides *within DNA* (particularly, in a purine-rich sequence context, such as in our DNAymes). Redox potentials of the 6MI heterocyclic base, dissolved in organic solvents, have been reported in the literature;[22] however, redox potentials of heterocyclic bases are highly sensitive to nearest neighbor effects (for instance, the oxidation potential of an isolated guanine in DNA is 1.20 V relative to NHE, while that of GGG is 0.64 V relative to NHE[27]) as well as to solvation status.

Currently, no high-resolution structure exists for the UV1C-TDP catalytic complex. However, extensive cross-linking and chemical probing studies, as well as data from experiments described here enable us to create a low-resolution conception of the functional components of UV1C-TDP, in three dimensions. Figure 6 shows schematic drawings of this conception, both as a side view (*i*) and a bottom-up (*ii*) view. The two classes of guanines able to serve as electron sources for photo-reactivation are shown in green and in blue. Guanines shown in pink do not directly contribute to photo-reactivation; however, they may serve as excitation energy conduits to guanines capable of directly participating in photo-reactivation.

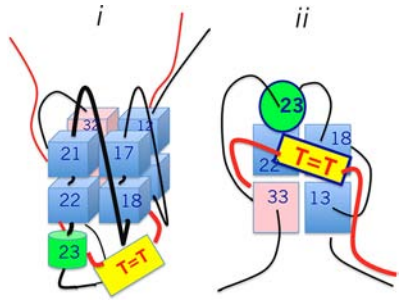

Figure 6. UV1C as a platform for constructing complex photolyase DNAzymes. Side view (*i*) and bottom-up view (*ii*) of a three-dimensional conception of the UV1C-TDP DNAzyme−substrate complex. The DNAzyme strand is shown in black, and the substrate in red. Blue and green guanines show alternative electron sources for the catalytic function of the DNAzyme. Pink guanines are those that do not participate directly in catalysis.

METHODS

Oligonucleotides. Oligonucleotides containing 6MI were purchased from Fidelity Systems. The DSS phosphoramidite was purchased from Glen Research. Both standard oligonucleotides and those containing DSS modifications were synthesized at the University of Calgary CORE DNA services. Two key DNA sequences were used in this study, UV1C: 5′-GGA GAA CGC GAG GCA AGG CTG GGA GAA ATG TGG ATC ACG ATT-3′ and TDP: 5′-AGG ATC TAC ATG TAT=TGT GTG CGT ACG AGT ATA TG-3′ (T=T refers to a special thymine dimer, which lacks the intervening phosphodiester). The single-stranded control oligonucleotide was LDP, a continuous piece of DNA with the same sequence as TDP, but lacking the latter's thymine dimer. The double-stranded control was TDP hybridized to a short splint oligonucleotide that was wholly complementary to the central portion of the TDP sequence.

Oligonucleotides were size purified in 8% denaturing polyacrylamide gels run in 50 mM Tris borate−EDTA (TBE) buffer. Following elution into TE (10 mM Tris, pH 8.0, 1 mM EDTA) buffer, the DNA was ethanol precipitated, washed twice with 70% v/v EtOH, and air-dried. The DNA pellets were redissolved in 40 μL of 5% acetonitrile (ACN) and 50 mM triethylamine acetate (TEAA), then loaded into Thermo Scientific Pepclean C18 spin columns. The columns were washed twice with 200 μL of 5% ACN, 50 mM TEAA, and the bound DNA eluted with 70% ACN, 50 mM TEAA. The ACN and TEAA were removed by ethanol precipitation, and the DNA pellet washed twice with 70% (v/v) ethanol, air-dried, and dissolved in TE buffer. DNA concentrations were determined by absorbance measurements taken in a Nanodrop spectrophotometer.

TDP was prepared as previously described,[9] and 5′-^{32}P-labeled with OptiKinase (Affymetrix) in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT. The kinased DNA was ethanol precipitated and gel purified, eluted overnight into TE buffer, and recovered by ethanol precipitation.

Figure S-6 shows that the UV−vis spectra of all nine G→6MI point mutants had the expected absorption peak at ∼345 nm wavelength, diagnostic of the presence of 6MI within each DNA construct. We used circular dichroism (CD) spectroscopy to confirm that all nine point mutants folded to a G-quadruplex (the DNAzymes gave complex CD spectra, owing to their folding to, in addition to a short G-quadruplex, extensive duplex, and other secondary structure elements—Figures S-4 and S-5), a functional test was carried out with the 6MI-containing DNAzymes—that they were able to photo-reactivate the TDP substrate with >320 nm light, and that this activity was notably higher in a sodium-containing buffer relative to a lithium-containing buffer (Figure S-3).

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CD Spectroscopy. Circular dichroism spectra of WT UV1C as well as of each mutant DNAzyme (all complexed with LDP), in addition to those of controls, were taken with a Jasco 810 CD spectrometer, using in a 0.1 cm path-length quartz cuvette (Starna). The DNAs (10 μM each) were dissolved in a solution of 20 mM Tris, pH 7.4, 2.0 mM EDTA at and 200 mM NaCl. Spectra were taken over the 200−400 nm wavelength range. The cuvette was rinsed thoroughly with ddH_2O in between different samples.

UV−Vis Spectroscopy. The UV−vis spectrum of each mutant DNAzyme, as well as of controls, was taken with a Cary spectrophotometer, using a 1 cm path-length quartz cuvette (Hellma). Spectra were taken from 230 to 450 nm.

Irradiation Sample Preparation. Each reaction took place in 100 μL of 20 mM Tris, 2 mM EDTA, pH 7.4, 200 mM NaCl (or LiCl), and 2 μM DNAzyme (or control DNA) and 2 nM 5′-^{32}P-labeled TDP. At these low DNA concentrations, the absorption of light was negligible along the laser path length. After all reagents had been added, except for the salt solution, the mixture was heated to 95 °C for 1 min, and then allowed to cool for 10 min, after which the NaCl was added from a stock solution to aid in the folding of the G-quadruplex. DNA solutions were prepared away from direct bright light and kept under darkness as far as possible.

Laser Irradiation. A Continuum Panther EX OPO laser, firing at 10 Hz, was first allowed to warm up for 30 min, at each of the wavelength settings used. The laser power at each wavelength was measured with a NIST-calibrated power meter and adjusted to 10 mW. To maximize pulse-to-pulse consistency, the laser was adjusted to its highest possible output and brought back to 10 mW with one or two beam splitters (Thor Labs). An electro-mechanically driven shutter (Thor Labs SH05 beam shutter) was used to ensure that irradiation times were accurate. The power meter was fixed in place to measure the laser power after the light had passed through the cuvette (Figure S-1). Laser power was monitored throughout the experiment, and upon observation of any irregularity, the sample was discarded and the irradiation repeated.

Each sample for irradiation was pipetted into a quartz micro-scale fluorescence cuvette, (Starna). The cuvette was shaken thoroughly to mix its contents prior to the drawing of each aliquot. A null aliquot was removed prior to irradiation, mixed with gel-loading dye, and stored in the dark at −20 °C. Each irradiated aliquot was likewise removed from the cuvette, and mixed immediately with denaturing dye to dissociate the DNAzyme−substrate complex, and stored in the dark at −20 °C. For laser irradiation, the cuvette holder was positioned such that the laser passed through the greatest length of the sample. The cuvette was rinsed thoroughly with ddH_2O between experiments.

Gel Analysis. Each DNA aliquot was brought to room temperature, mixed thoroughly, and run in 8% denaturing polyacrylamide gels run in 50 mM TBE buffer. Samples were loaded to ensure at least 10 counts per second in each lane. Gels were exposed to phosphor screens, which were then scanned with a Typhoon phosphorimager (GE). Peak intensities were quantitated using the ImageJ software included with the phosphorimager. The integrated area of a "repaired" band was divided by the sum of the "repaired" and "unrepaired" areas of that particular lane, then multiplied by 100 to give a percentage value for repair. Any small initial amount of repaired DNA, at time = 0 (corresponding to no laser irradiation), was subtracted from each repair percentage in the irradiated samples.

Kinetic Analysis. Initial rates were determined by regression through data points, measured as a function of time, that were in the linear range (i.e., represented <10% overall repair). Typically, five data points were measured within the linear response range. Duplicate samples were irradiated in every case, to estimate the error in each measurement. Figure S-8 shows a typical plot of the fraction of thymine dimer repaired as a function of irradiation time.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary data on the experimental setup and on characterization of G→6MI mutant DNAzymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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