

Turning the 10–23 DNAzyme On and Off with Light

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DNAs with catalytic function, or DNAzymes, have become important tools for a variety of biochemical applications.^[1] Some of the most widely known DNAzymes, the 8–17 and the 10–23, were evolved by *in vitro* selection for RNA cleavage^[2] as a way of inhibiting gene expression.^[3] Other DNAzymes have been used for catalyzing chemical reactions,^[4] as biosensors^[5,6] and in nanodevices.^[7–9] Being able to photomodulate the function of DNAzymes with high spatial and temporal resolution could be useful for many applications in biotechnology and biology.

Photoactivatable (also known as “caged”) oligonucleotides have been used primarily to study gene expression,^[10] and more recently, have also been used for controlling PCR product generation,^[11] RNA folding,^[12] oligonucleotide mismatch detection^[13] and conformation switches in nanomachines.^[14] The focus of the current study is the 10–23 DNAzyme, a Mg^{II}-dependent enzyme that catalyzes RNA cleavage at a pyrimidine–purine junction—preferentially AU. The enzyme consists of a 15-base catalytic loop (GGCTAGCTACAACGA) and binding arms 6–12 bases long that hybridize to the target RNA. We report the design of caged 10–23 DNAzymes that either inactivate by breaking apart upon UV irradiation (Figure 1B, C) or are blocked from hybridizing before irradiation (Figure 1D, E) depending on the placement of a light-induced strand break (Figure 1F).

Caged versions of the 8–17^[15] and 10–23 DNAzymes^[16] have been developed previously by using phosphoramidite chemistry to incorporate photoactivatable bases at key positions in the enzyme. These modifications disrupted DNAzyme function until removal with UV light, which turn gene expression from “on” to “off”. Reversible 8–17 and 10–23 DNAzymes were created by replacing key bases with a nucleotide modified with the photoisomerizable azobenzene group, which adopts the *cis* conformation under UV irradiation and reverts to the *trans* isomer in visible light.^[17] The 10–23 DNAzyme showed higher catalytic activity with *cis*-azobenzene, whereas the 8–17 DNAzyme was more active in *trans* form. This approach offers photoreversibility, but requires synthesis of azobenzene-modified nucleotides and remains to be optimized for bidirectional control.

Here, we used a 10–23 DNAzyme targeting the VEGFR2 receptor mRNA previously characterized by Zhang et al.^[3c] The strand break was engineered site-specifically by using a photo-cleavable spacer (PS, Figure 1F) that is commercially available

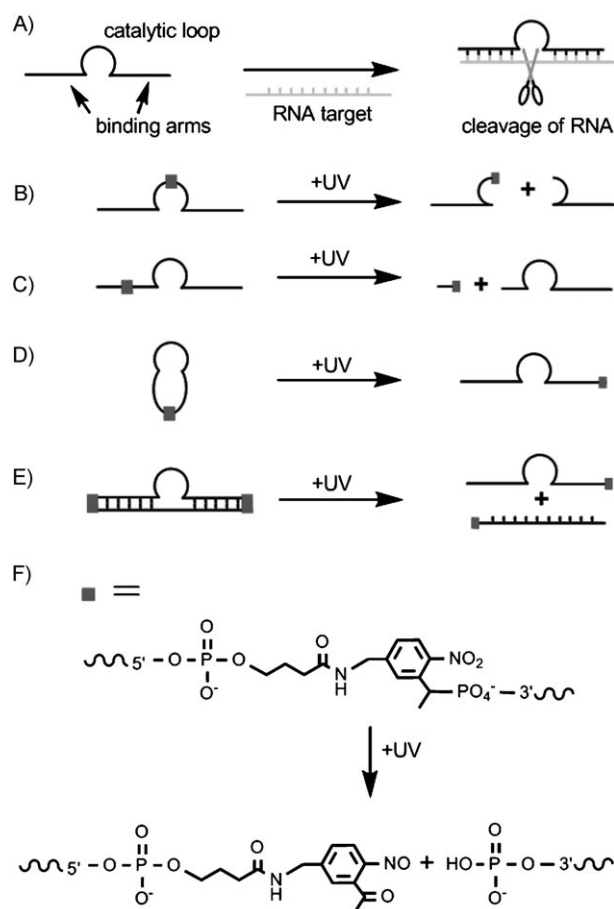


Figure 1. A) General structure and function of the 10–23 DNAzyme; B) and C) the photocleavable spacer (PS, square) within the DNAzyme core and binding arms causes the enzyme to fragment upon UV photolysis; D) and E) circularized DNAzymes have impaired function until photolysis of PS restores the linear form; F) structure of PS and product of photolysis.

as a phosphoramidite (Glen Research) and can be incorporated in a sequence anywhere during solid-phase synthesis.

The first site for PS incorporation was the 10–23 catalytic core (Figure 1B), which has been well studied through base substitutions and deletions. Changes to bases 7–12 of the core tend to cause the smallest loss of catalytic activity.^[18] In particular, thymine in the eighth position (T8) is the most flexible to substitutions by all three other bases and inosine, as well as to deletion.^[19] Cytosine in the seventh position (C7) can also be deleted with moderate loss of activity, and a double deletion mutant lacking both C7 and T8 retains some activity.^[19] We, therefore, designed the first three caged DNAzymes and replaced either T8 (Dz1), C7 (Dz2), or both C7 and T8 (Dz3) with PS (Table 1).

The DNAzymes were tested for their ability to catalyze cleavage of a 28-nucleotide (nt) radiolabeled RNA substrate *in vitro*,

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Table 1. DNAzyme sequences, data fitting, and melting temperatures. First-order rate constants (k) were fit to $[S] = A(1 - e^{-kt})$, where $[S]$ is the fraction of substrate uncleaved at time t , and A is the percentage of total RNA cleaved as time approaches infinity. Binding arms are in lowercase and catalytic loop in uppercase; PS indicates the photocleavage site.

	Sequence	k [min^{-1}]		Photo-modulation ^[a]	A [%]		T_m [$^{\circ}\text{C}$]
		-UV	+UV		-UV	+UV	
Dz0	tgctctccaGGCTAGCTACAACGAcctgcacct	0.24(\pm 0.03)	0.21(\pm 0.02)	1:1	77(\pm 2)	80(\pm 2)	49
Dz1	tgctctccaGGCTAGC-PS-ACAACGAcctgcacct	0.089(\pm 0.003)	0.039(\pm 0.003)	2:1	70(\pm 1)	76(\pm 3)	49
Dz2	tgctctccaGGCTAG-PS-TACAACGAcctgcacct	inactive	inactive	n.a.	-	-	49
Dz3	tgctctccaGGCTAG-PS-ACAACGAcctgcacct	inactive	inactive	n.a.	-	-	49
Dz4	tgctctccaGGCTAGCTACAACGAcct-PS-cacct	0.055(\pm 0.007)	0.032(\pm 0.01)	2:1	53(\pm 3)	21(\pm 4)	39
Dz5	tgctc-PS-ccaGGCTAGCTACAACGAcctgcacct	0.12(\pm 0.02)	0.077(\pm 0.01)	2:1	81(\pm 3)	31(\pm 1)	39
Dz6	tgctc-PS-ccaGGCTAGC-PS-ACAACGAcctgcacct	0.069(\pm 0.002)	inactive	on \rightarrow off	74(\pm 1)	-	36
Dz7	tgctctccaGGCTAGCTACAACGAcctgcacct <div style="text-align: center;"> PS </div>	0.022(\pm 0.007)	0.21(\pm 0.02)	1:10	58(\pm 10)	79(\pm 1)	43
Dz8	tgctctccaGGCTAGCTACAACGAcctgcacct <div style="text-align: center;"> PS-cgagaggtaggacgtgg-PS </div>	inactive	0.024(\pm 0.009)	off \rightarrow on	-	56(\pm 10)	n.d. ^[c]
Dz1a	tgc tct cca GGC TAG C	0.10(\pm 0.02) ^[b]	n.a.	n.a.	51(\pm 2)	n.a.	39
Dz1b	5'-PO ₄ ²⁻ -ACA ACG Acc tgc acc t						39

[a] Photomodulation is the ratio of rate constants (k) before and after UV photolysis. [b] Dz1a and Dz1b were inactive when tested alone; k value is for a 1:1 mixture of the two strands. [c] The internal melting temperature of Dz8 alone was 74°C , so its melting temperature with the target RNA was not determined.

GCGCGAGGUGCAGGAUUGGAGAGCAAGGC. Bases targeted by the binding arms are underlined and the RNA cleavage site is highlighted in bold. Irradiation of the DNAzymes was carried out for 20 min with a UV transilluminator (9 mW at peak intensity, 365 nm). Gel electrophoresis of the DNAzymes containing PS anywhere in the sequence showed that after 20 min there was no intact DNAzyme remaining (see the Supporting Information).

Kinetic data from the RNA cleavage experiments were fit by a single exponential decay function, $[S] = A(1 - e^{-kt})$, where $[S]$ is the fraction of RNA uncleaved at time t . Apparent first-order rate constants (k) were determined, and A represents the percentage of RNA cleaved as time approaches infinity (Table 1). Near-UV irradiation did not appear to damage the unmodified DNAzyme, Dz0, as its rate coefficient was essentially unchanged by irradiation ($k = 0.24 \text{ min}^{-1}$ before and $k = 0.21 \text{ min}^{-1}$ after) with similar percentages of total RNA cleavage ($A = 77$ and 80%). Of the photoactive DNAzymes tested, Dz2 and Dz3 showed no cleavage after 1 h incubation, even without irradiation; this indicates that replacement of C7 with PS abolishes activity.

Replacing T8 with PS within the catalytic core produced an active enzyme (Dz1) with lower efficiency ($k = 0.089 \text{ min}^{-1}$). Surprisingly, the enzyme activity decreased only twofold ($k = 0.039 \text{ min}^{-1}$) upon irradiation. Furthermore, the unirradiated and irradiated enzymes showed similar amounts of total RNA cleavage at later time points ($A = 70$ and 76%). To investigate these results for Dz1, the two halves of the DNAzyme were synthesized and tested. The 5' half of the DNAzyme, Dz1a, consisted of the 5'-binding arm and bases 1–7 of the catalytic loop. Dz1b consisted of bases 9–15 of the catalytic loop and the 3'-binding arm. Dz1b was also phosphorylated at its 5' end so that it would be the same as the product of Dz1 photolysis. When Dz1a, Dz1b, and RNA target were mixed simultaneously

in the same reaction, significant RNA cleavage was observed ($k = 0.10 \text{ min}^{-1}$), which shows that the two halves can reconstitute the active enzyme while lacking a phosphodiester linkage. After 1 h, the mixture of Dz1a and Dz1b cleaved 61% of the target RNA, almost as much as the 70% RNA cleaved by full-length Dz1 (Figure 2B).

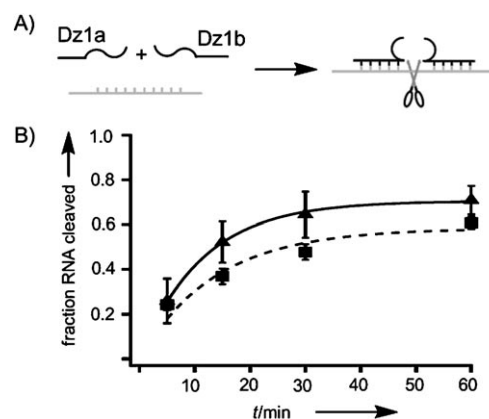


Figure 2. A) Scheme showing the two DNA halves and RNA target mixed together to form the active enzyme; B) RNA cleavage versus time for Dz1 (—) and the mixture of the two halves of the enzyme, Dz1a and Dz1b (-----).

The Dz1a+Dz1b mixture performed as well as Dz1 in the early time points; this suggests that formation of the catalytic site occurs rapidly. Another DNAzyme consisting of two noncovalently linked oligonucleotides has been made previously by incorporating half a DNA aptamer in each sequence.^[6] In the presence of hemin, the two halves of the aptamer assemble into a G quadruplex and the split DNAzyme becomes active. For the split 10–23 DNAzyme, further study needs to be con-

ducted to determine whether the metal-binding properties and intramolecular contacts play roles in maintaining the structure and function of the enzyme after photolysis.

A second set of DNAzymes was then designed to incorporate strand breaks within the binding arms (Figure 1 C). PS was placed three bases downstream from the catalytic site in the 3'-binding arm (Dz4) and three bases upstream from the catalytic site in the 5'-binding arm (Dz5). A guanine in Dz4 was replaced while a thymine was replaced in Dz5. The sensitivity of DNAzyme arm length has been found to be highly sequence dependent.^[20] Shortening each arm for different DNAzymes can decrease or increase the cleavage rate by varying degrees, making it difficult to predict whether phototruncation should affect Dz4 and Dz5.

Substitution of PS in each binding arm produced enzymes with very different activities. Dz5 was slower than Dz0 ($k = 0.12 \text{ min}^{-1}$) but still cleaved the same amount of RNA as Dz0 during the experiment ($A = 81\%$). Dz4 showed even less activity ($k = 0.055 \text{ min}^{-1}$ and $A = 53\%$). Upon irradiation of Dz4 and Dz5, the activities of both decreased twofold. However, the binding arm truncations were clearly not sufficient to inactivate the enzyme.

To increase the degree of photomodulation, a new DNAzyme (Dz6) was designed to combine the catalytic core and binding arm modifications, with PS at both T8 and in the 5' binding arm. The robustness of the 10–23 DNAzyme to function as two halves or with shorter binding arms seemed to necessitate splitting the enzyme into three separate parts to achieve inactivation. Unphotolyzed Dz6 showed similar activity to Dz1 ($k = 0.069 \text{ min}^{-1}$ and $A = 74\%$) and, unlike the other DNAzymes, RNA cleavage after UV irradiation was completely abolished (Figure 3 A).

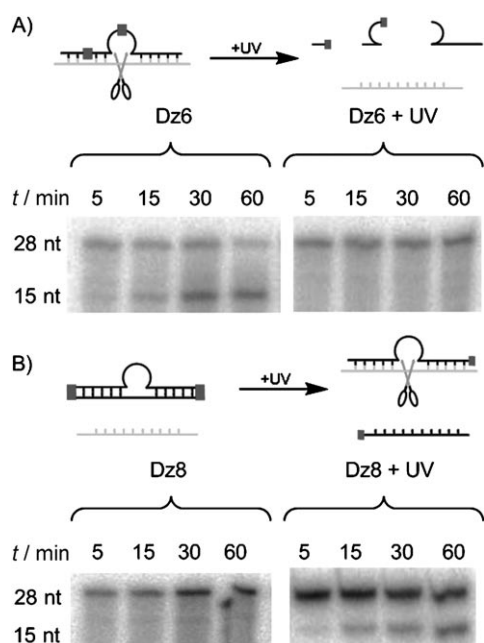


Figure 3. A) Dz6 hydrolyzes the radiolabeled 28-nt RNA substrate to form a 15-nt product. In a parallel experiment, no Dz6 activity is seen after UV irradiation; B) in contrast, Dz8 shows no RNA hydrolysis until UV irradiation restores activity.

Having successfully made a DNAzyme that switched from “on” to “off” upon photolysis, we worked to develop a DNAzyme that was turned “on” by UV irradiation. To prevent the caged enzyme from functioning normally, we tried to block DNA hybridization to the RNA target. Modulating hybridization by using photocleavable groups has been accomplished previously by several methods. One strategy has been to incorporate a number of photocleavable groups within the backbone to block binding sterically.^[21] A similar approach has used caged nucleobases to prevent base pairing in the sequence.^[22] Recently, a single photocleavable linker has been used to join the oligonucleotide to a complementary blocking strand that will dissociate upon photolysis.^[23]

In order to achieve more complete inhibition of hybridization, the DNAzymes were circularized with the PS included in the sequence. Previously, circular DNAzymes have been made by enzymatic ligation^[24] or cloning into an expression vector^[25] in order to make them more nuclease resistant and improve cellular delivery. These DNAzymes were 70 and 100 nt in length, thus their function was not impeded after circularization. In our design, we hypothesized that joining the 5' and 3' ends of this shorter 33-nt DNAzyme should create a compromised enzyme that gains activity upon UV irradiation and linearization.

For Dz7, PS was incorporated between the 5' and 3' ends of the DNAzyme to create a circular structure (Figure 1 D). Because it is not known whether the structure of PS would be recognized by the ligase, the ligation site was chosen to be at a distance, between guanine G6 and cytosine C7 of the catalytic loop. The linear sequence was synthesized by using standard phosphoramidite chemistry with the phosphorylated G6 located at the 5' end and C7 at the 3' end, placing PS in the middle. The two ends were joined in 40% yield by using CircLigase, a single-stranded DNA ligase optimized for circularization of oligonucleotides with 5' phosphate and 3' hydroxyl groups. Any linear DNA remaining was digested by using exonuclease I.

Dz7 was assayed for its ability to cleave RNA under the same conditions used for Dz1–6. Before photolysis, Dz7 cleaved RNA slowly, $k = 0.022 \text{ min}^{-1}$ (Table 1). After photolysis, activity was restored to the level observed for the unmodified enzyme Dz0, creating a tenfold enhancement. The melting temperature of Dz7 hybridized to the RNA target is 43°C , which although 6°C lower than the melting temperature of the linear DNAzyme (Table 1), indicates that the circularized DNA still hybridized to its target under the conditions of the enzymatic assay.

To ensure that hybridization could not occur with the RNA, we applied the circularization technique to create a DNAzyme (Dz8) that incorporated a self-complementary blocking strand. PS was incorporated at two positions: joining both the 5' and 3' ends of the DNAzyme to a DNA sequence identical to bases 7–23 of the RNA substrate, including the bulged A residue (Table 1). Like Dz7, the initial linear sequence of DNA was made by phosphoramidite chemistry including a 5' phosphate with the ligation site between G6 and C7. PS was again incorporated in the middle of the linear sequence followed by the

complementary region of DNA and a second photocleavable spacer. It was then enzymatically circularized under the same conditions as Dz7, with similar yields, to produce a circular structure with self complementarity (Figure 1E).

In contrast to Dz7, Dz8 cleaved no RNA in a 1 h time course prior to UV irradiation. The complementary DNA successfully prevented Dz8 from having DNAzyme activity (Figure 3B). Postphotolysis, the enzyme gained modest activity ($k = 0.024 \text{ min}^{-1}$) and cleaved more than half of the RNA target during the course of the experiment ($A = 56\%$). As with Dz6, reduced activity in the "on" state was traded for a complete shutoff of activity in the "off" state. Importantly, with Dz6 and Dz8, complete photomodulation was successfully achieved to create DNAzymes that switch "on" or "off".

Caged DNAzymes of any sequence that will either activate or inactivate upon UV irradiation can be made by using commercially available reagents without the need for synthesizing photoactive nucleobases. Photocleavable linkers similar to PS have been used for a variety of applications involving DNA strand breaks,^[12a,24a,26] such as the study of strand repair, end labeling of oligonucleotides, or control of hybridization. This work provides a novel use for PS and could be extended to other linkers.

In conclusion, these approaches should facilitate the development of caged DNAzymes for numerous biological applications. The circularization technique described provides a method for completely blocking oligonucleotide hybridization, which has many potential uses in biotechnology. In addition, this work demonstrates the robustness of the 10–23 DNAzyme, which can tolerate the chemically dissimilar substitution of PS for a nucleotide. It was also found to function as a binary DNAzyme divided within the catalytic core.

Experimental Section

RNA cleavage assay: Single turnover assays were performed at 37 °C with DNAzyme (0.83 μM) and ³²P-labeled RNA substrate (0.083 μM) in MgCl₂ (10 mM), Tris pH 7.5 (10 mM), and NaCl (83 mM). For experiments in which the DNAzyme was photolyzed, the reaction mixture without the RNA target was irradiated in an open 0.2 mL tube by a UV transilluminator (9 mW cm⁻² at 365 nm) and the reaction was then initiated by addition of the RNA. Aliquots (5 μL) were removed at regular time intervals and the reaction was quenched with RNA loading buffer II (Ambion). The aliquots were run on a urea (7 M) polyacrylamide gel (20%) at 300 V for 40 min. Gels of RNA digests were imaged by using an Amersham Biosciences Storm 840 phosphorimager. Gels were analyzed by using TotalLab Software (Nonlinear Dynamics) to detect the band intensities and correct for background by using the rubber-band subtraction function. Then the ratio of intensities of the uncleaved and cleaved RNA bands was calculated. First-order rate constants (k) were fit to a single exponential function by using Igor (WaveMetrics), $[S] = A(1 - e^{-kt})$, where $[S]$ is the fraction of substrate uncleaved at time t , and A is the percentage of total RNA cleaved as time approaches infinity.

DNAzyme circularization: Dz7 and Dz8 were synthesized as linear sequences by standard phosphoramidite chemistry and chemically phosphorylated during synthesis. The linear sequences were, respectively: GCTACAACGACCTGCACCT-PS-TGCTCTCCAGGCTA and

GCTACAACGACCTGCACCT-PS-GGTGCAGGATGGAGAGC-PS-TGCTCTCCAGGCTA. Circligase (Epicentre, Madison, WI, USA) was used to create a phosphodiester linkage between these two ends by using the manufacturer's protocols with DNA (8 μM) and ligase (5 μM). After the reaction, exonuclease I was used to digest any remaining linear DNA. The product was purified by phenol/chloroform extraction and desalted on a NAP-10 column. Analysis by gel electrophoresis showed the circular product migrating more slowly than the linear sequence, and returning to its original position upon UV irradiation (see the Supporting Information).

Thermal denaturation methods: Solutions for the thermal denaturation studies contained 19-base RNA (1 μM) with the AU cleavage site replaced with GU (AGGUGCAGGGUGGAGAGCA) and the complementary DNAzyme in Tris-HCl, pH 8.0 (10 mM) and NaCl (100 mM). Melting studies were conducted on a Beckman Coulter DU800 UV/Vis spectrophotometer equipped with a programmable Peltier temperature controller. Samples were monitored at 260 nm while being heated or cooled at a rate of 1.0 °C min⁻¹, with a 1 min hold per degree Celsius. Melting temperatures were determined from the peak of the first derivative plot of A_{260} versus temperature.

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