RNA Cleavage

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Reversible Photocontrol of Deoxyribozyme-Catalyzed RNA Cleavage under Multiple-Turnover Conditions**

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Light is a highly effective and well-established bioorthogonal trigger by which the chemical or biochemical reactivities of nucleic acids can be localized in time and space.[1] Typically, photoactivation results from the irreversible removal of a masking group from strategic functionalities and has been utilized for the construction of DNA arrays, the study of RNA

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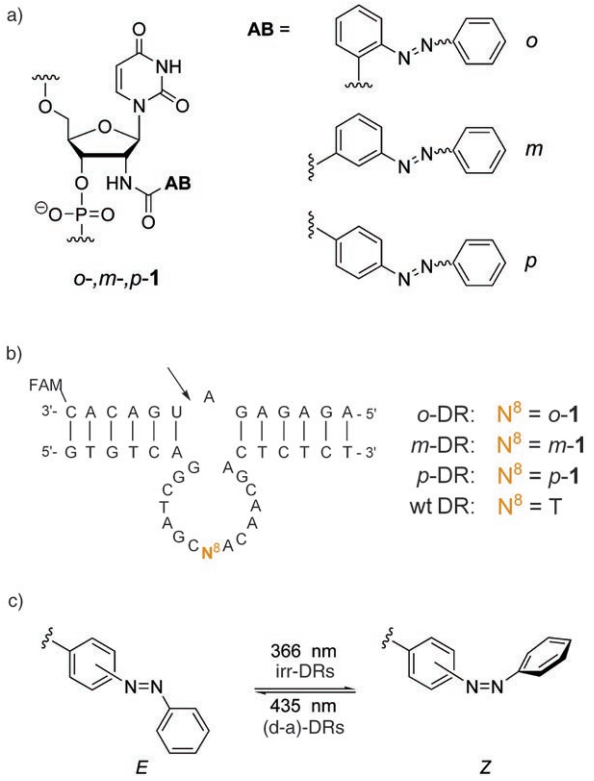
cleavage and folding, the triggering of deoxyribozyme or aptamer activities, and also for the in vivo regulation of gene expression.[2] Alternatively, reversible optical control of DNA recognition has been reported with backbone-substituted azobenzene derivatives.[3] $E \rightarrow Z$ photoisomerization of azobenzenes occurs with high quantum yields at 330–370 nm, is not particularly sensitive to the environment, is fatigue-resistant, and leads to large conformational and polarity changes.[4] Modest discrimination in activities between the so-called irradiated (mainly Z) and more active dark-adapted (mainly E) states of papain modified by nonspecific attachment of azobenzenes to surface lysines was reported in 1991.[5] More recent reports have demonstrated that localization of azobenzenes at strategic residues of transmembrane proteins[6] or DNA-binding oligopeptides,[7] in which the E isomer is inactive, enables highly effective activity modulation by light.

To date, a single report of photoswitchable 8–17 deoxyribozyme-mediated RNA cleavage has been made.[8] However, in this report, only single-turnover behavior with a 200-fold excess of the deoxyribozymes over the substrate is described, highly attenuated activities are shown, and the incorporation of two azobenzene units is required for significant (up to 5.4-fold) light-induced rate modulation. Both the 8–17 and 10–23 RNA-cleaving deoxyribozymes were isolated by in vitro selection from the same library,[9] and have found application in a wide range of roles, for example, as sensors for bacterial rRNA, metal ions, and effector sequences, and for the in vivo regulation of specific mRNA levels.[10] In particular, 10–23 deoxyribozymes can cleave any target purine–pyrimidine motif and have wide tolerance for modifications in the binding arms or catalytic core, thus enabling both substrate affinity and serum stability to be significantly enhanced by chemical modification.[11,12]

Herein, we report the first photoswitchable RNA cleavage by nucleic acid catalysts under multiple-turnover conditions, in which only a single nonconserved residue is substituted by novel, readily accessible nucleotide analogues. Cleavage rates are enhanced up to ninefold following irradiation at 366 nm, thus reaching the rates observed for the unmodified deoxyribozyme.

We prepared 10–23 deoxyribozymes in which a single nucleotide (T8) within the catalytic core was replaced by a 2′-deoxyuridylate analogue. *Ortho*-, *meta*-, or *para*-phenylazobenzoyl moieties were appended to this residue through a 2′-amido linkage (Scheme 1 a). This site was chosen as deletion of T8 or substitution by 2′-*O*-methyluridine has previously been shown to only minimally perturb deoxyribozyme activities.[11] The deoxyribozyme constructs were engineered with short binding arms to promote product release under multiple-turnover conditions.

Modified nucleoside precursors were prepared by reaction of the protected 2′-aminonucleoside[13] with the *N*-hydroxysuccinimidyl ester of the appropriate azobenzene,[14] and subsequently phosphitylated under standard conditions. These precursors were incorporated into model octamers (ACC**1**GGTA) and also 10–23 deoxyribozyme sequences (Scheme 1 b) by automated solid-phase synthesis with extended reaction times for introduction of the modified



Scheme 1. a) Azobenzene-modified uridylates used in this study. b) The 10–23 deoxyribozymes prepared (FAM = 6-fluoresceinyl; cleavage site indicated by arrow). c) Photoisomerization of the azobenzene unit (irr-DR and (d-a)-DR refer to irradiated and dark-adapted 10–23 deoxyribozymes, respectively).

residue. Coupling yields as monitored by trityl release were greater than 98%. After deprotection under anhydrous conditions, the oligonucleotides were purified by reversed-phase HPLC and their identities were confirmed by MALDI-TOF analysis.

The photoisomerization of oligodeoxynucleotide-appended azobenzenes under irradiation at 366 nm (Scheme 1 c) was investigated by UV/Vis spectroscopy of the model octamers. When the photoswitches were in the thermally stable E configuration, the spectra showed local maxima at 330 (*o*-**1**), 321 (*m*-**1**), and 329 nm (*p*-**1**) that were typical of azobenzene π–π* transitions. Irradiation at 366 nm led to loss of these absorption maxima, with photostationary states being achieved within 8 min. These results were reproduced with deoxyribozymes (DRs) appended to azobenzene, and the $E \rightarrow Z$ conversion yields were measured immediately by HPLC. Peak quantification at 260 nm was used to determine the level of E and Z isomers either directly for *m*-DR and *p*-DR, or by inference using the model octamer sequence containing *o*-**1**. After irradiation, $E \rightarrow Z$ conversion yields of 86% for irr-*o*-DR, 75% for irr-*m*-DR, and 61% for irr-*p*-DR were thus determined. At 26 °C the *ortho* and *meta* photoswitches within both the model sequences and the deoxyribozymes were stable toward $Z \rightarrow E$ thermal back-isomerization. In contrast, *para* photoswitches underwent more rapid thermal reisomerization at this temperature. In all

cases $Z \rightarrow E$ photoisomerization was complete following irradiation at 435 nm for 2 min.

Both azobenzene-modified and unmodified (wild-type) deoxyribozymes (wt DRs) catalyzed the site-specific cleavage of a 13-mer oligoribonucleotide substrate labeled at its 3′ terminus with a fluoresceinyl moiety (FAM) to yield a labeled hexamer and a 2′,3′ cyclic phosphate-terminated heptamer. The cleavage reactions were thus resolved, visualized, and quantified in a polyacrylamide gel electrophoresis (PAGE) assay (Figure 1a). Deoxyribozyme solutions were exposed to light at 366 nm for 10 min or at 435 nm for 2 min, and reactions were initiated by the addition of substrate RNA followed by incubation at 26 °C in the absence of light. To assess the cleavage activity of the more thermally labile irr-*p*-DR, continuous irradiation at 366 nm was performed during the assay.

Under multiple-turnover conditions, irradiated azobenzene–deoxyribozymes maintained essentially wild-type activities; thus, irr-*o*-DR, irr-*m*-DR, and irr-*p*-DR showed cleavage rates of 100, 90, and 50%, respectively (Figure 1b). In contrast, RNA cleavage rates by dark-adapted (d-a) deoxyribozymes were considerably attenuated. The $k_{irr}/k_{d\text{-}a}$ ratios in this assay were determined to be 9:1 for *o*-DR and *p*-DR, and 8:1 for *m*-DR (Figure 1c). Photocontrol of RNA cleavage by deoxyribozyme–azobenzene conjugates was also demonstrated by using an unlabeled RNA substrate and reversed-phase HPLC analysis.[14] The effects observed in these assays compare well with the results from the PAGE analyses. Thus, the relative rates of substrate cleavage by irr-*o*-DR and irr-*m*-DR are both the same as for the unmodified deoxyribozyme wt DR, and irr-*p*-DR shows 44% of the wild-type activity. Dark-adapted deoxyribozymes give significantly less conversion than the irradiated constructs; $k_{irr}/k_{d\text{-}a}$ discrimination factors of 6 for *o*-DR and 5 for *m*-DR and *p*-DR constructs were observed.

The effect of photoswitching upon catalysis by the azobenzene-conjugated deoxyribozymes was also demonstrated to be reversible under multiple-turnover conditions with either 5 mol% (*o*-DR or *m*-DR) or 10 mol% (*p*-DR) deoxyribozyme (Figure 2). After initial irradiation of deoxyribozyme solutions at 366 nm the reactions were initiated by addition of substrate, and RNA cleavage rates comparable to those previously described were observed. After 120 min the reactions were irradiated at 435 nm for 2 min to afford the dark-adapted deoxyribozymes which gave rise to significantly retarded reaction rates, although these were higher than those previously observed using purified *E*-DRs. Thus, $k_{irr}/k_{d\text{-}a}$ discrimination factors of 3.6 for *o*-DR, 3.8 for *m*-DR, and 4.1 for *p*-DR were observed. This difference might be accounted for by perturbation of the $Z \rightarrow E$ photoisomerization process[4] in the presence of substrate RNA or kinetic

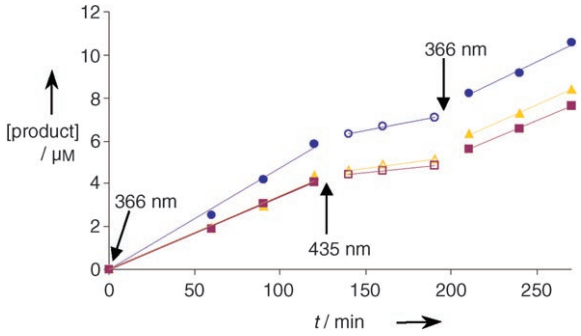


Figure 2. Reversibility of RNA (20 μM) cleavage by 10–23 deoxyribozymes *o*-DR (1 μM), *m*-DR (1 μM), and *p*-DR (2 μM) under multiple-turnover conditions. Key: ○ (d-a)-*o*-DR; ● irr-*o*-DR; △ (d-a)-*m*-DR; ▲ irr-*m*-DR; □ (d-a)-*p*-DR; ■ irr-*p*-DR.

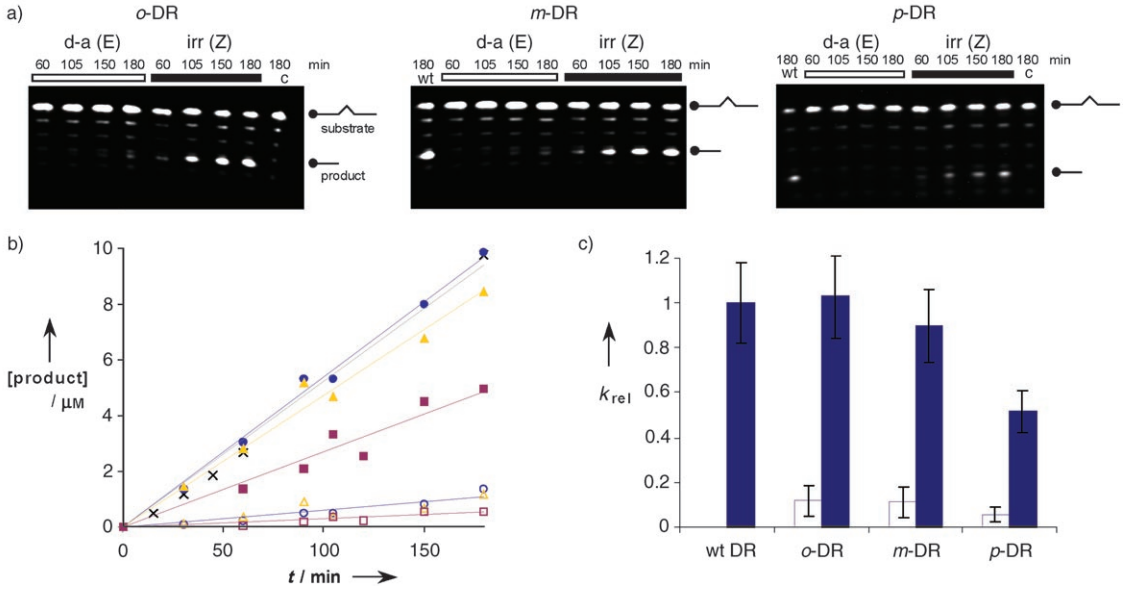


Figure 1. RNA (20 μM) cleavage by 10–23 deoxyribozymes *o*-DR, *m*-DR, *p*-DR, and wt DR under multiple-turnover conditions (10:1 RNA/deoxyribozyme). a) PAGE analysis of 3′-FAM-labeled RNA substrate strands (wt: wt DR RNA cleavage; c: control without DR). b) Quantitative analysis of product formation; key: ○ (d-a)-*o*-DR; ● irr-*o*-DR; △ (d-a)-*m*-DR; ▲ irr-*m*-DR; □ (d-a)-*p*-DR; ■ irr-*p*-DR; × wt DR. c) k_{rel} values for dark-adapted (open bars) and irradiated (filled bars) deoxyribozymes normalized to the unmodified deoxyribozyme reaction.

folding traps, which do not respond to this isomerization.[15] However, the initial cleavage rates were recovered following irradiation at 366 nm (Figure 2).

Large differences in the steric demands and hydrophobic characters of the *E* and *Z* isomers of *para*-azobenzene residues attached to biomolecules are well-described,[16] but we are unaware of any other report in which the *ortho* isomers give similar activity switching. Preliminary NMR spectroscopic investigations indicate that both *E* and *Z* isomers of *p*-**1** reside in the C2′-*endo* furanoside pucker typical of 2′-amido deoxyribonucleoside analogues and their unmodified congeners, and so the active conformation of the catalytic core containing the photoswitch may be modulated in some other fashion.

Our demonstration of photomodulated deoxyribozyme-catalyzed RNA cleavage under multiple-turnover conditions is of particular interest as the irradiated "on" state maintains wild-type cleavage rates. The novel analogues described herein enable incorporation of azobenzene moieties with readily accessible nucleoside derivatives, which have the potential to maintain essential base contacts and the biological activity of nucleic acids. We envisage that the ability to reversibly modulate the catalytic RNA cleavage rates of the 10–23 deoxyribozyme by light will add a useful tool to the repertoire of regulatory biocatalysts. We are currently working toward the development of light-programmable conformational switches within DNA and RNA,[17] and their application to the spatiotemporal control of gene expression and array-based computation.[18]

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