Enhancement of RNA cleavage activity of 10–23 DNAzyme by covalently introduced intercalator†

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By introducing an intercalator through D-threoninol to the 10–23 DNAzyme at the junction between its catalytic loop and the binding arm, the RNA cleavage activity was greatly improved.

The 10–23 DNAzyme, captured from a DNA pool of random sequences by *in vitro* selection, is an RNA cleaving oligodeoxyribonucleotide composed of two binding arms (arm I and arm II) and one 15 nt-long catalytic loop (Fig. 1).[1,2] As an antisense agent, it displays high activity and specificity under physiological conditions, and shows high chemical and biological stability in comparison with other antisense nucleic acids like ribozyme. In addition, it has a broad target range with its only sequence requirement being 3′-pyrimidine–purine-5′ (YR).[1a] This DNAzyme has already been applied to the suppression of a number of specific genes *in vitro* as well as *in vivo*.[3,4] However, Cairns *et al.* have recently found that cleavage activity of the 10–23 DNAzyme depends greatly on the sequence at the YR cleavage site: UA = UG ⩾ CG ≫ CA.[5] For the RNA substrate with a CG or CA sequence at the cleavage site, efforts should be made for expanding its utility. In this study, we report that the activity of RNA cleaving 10–23 DNA enzyme is improved by covalently introducing an intercalator, such as azobenzene, 2-stilbazole, pyrene, and anthraquinone. The intercalator is attached *via* an amide bond to D-threoninol, which inserts into the backbone of DNA with typical phosphoramidite chemistry. Effects of the intercalator structure and the type of linkage between DNA backbone and the intercalator are also demonstrated.

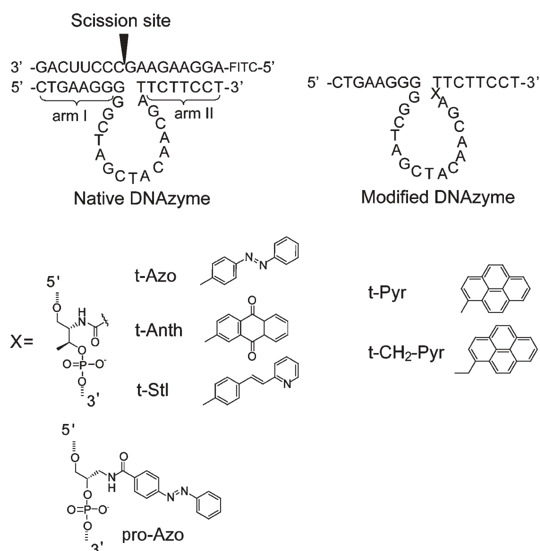

Fig. 1 DNAzymes, substrate RNA, and intercalators used in the present study.

The 17 mer RNA substrate used here (see Fig. 1 for the sequence) is a part of abnormal L6 *BCR-ABL* fusion mRNA, which is an antisense target for curing chronic myelogenous leukemia (CML).[3,6,7] Its 5′ terminal is labelled with fluorescein-4-isothiocyanate (FITC). This short RNA has only one YR sequence (CG), which is difficult to be efficiently cleaved by native 10–23 DNAzyme.[3,5] Fig. 2 depicts the typical time course of RNA cleavage by the corresponding DNAzymes (see Fig. 1 for the sequence).[8] As shown by the dotted line, unmodified native

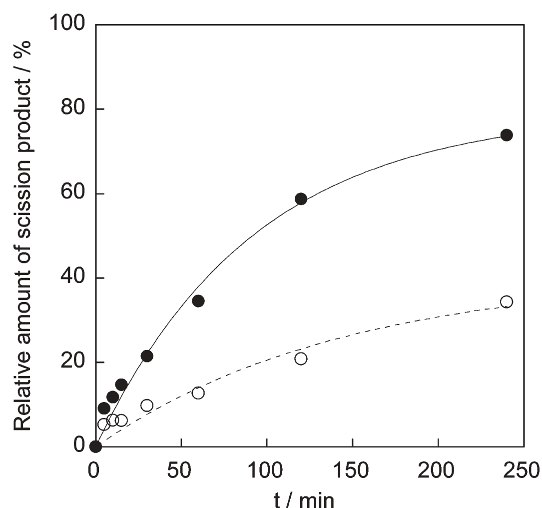

Fig. 2 Time courses of the RNA cleavage by the native (open circles) and t-Azo-tethered (closed circles) DNAzymes under single turnover conditions at 37 °C in the presence of 1.0 M NaCl and 10 mM $MgCl_2$ at pH 7.5 (50 mM Tris buffer). Concentrations of DNAzyme and substrate RNA were 10 μM and 0.8 μM, respectively.

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† Electronic supplementary information (ESI) available: Experimental procedures for the syntheses of phosphoramidite monomers of t-Anth, t-Stl, and pro-Azo, actual PAGE patterns of Fig. 2, effect of the position of the X residue on the cleavage activity, time course of RNA scission by t-Anth-tethered DNAzyme. See DOI: 10.1039/b611078a

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DNAzyme cleaved the RNA rather slowly: the obtained pseudo-first order rate constant (k_{obs}) was 2.2×10^{-3} min^{-1}, which is 10–100 fold smaller than the UA or UG sequence as reported by Cairns *et al.*[5] For improving the activity, we modified this DNAzyme by tethering an intercalator *via* D-threoninol linker at various positions in the DNAzyme.[9,10]‡ When a *trans*-azobenzene was tethered at the junction between the catalytic loop and arm II (X = t-Azo in Fig. 1), RNA was cleaved much more efficiently than with the unmodified DNAzyme (compare solid line with dotted one in Fig. 2): k_{obs} of azobenzene-tethered enzyme (X = t-Azo) was 6.5×10^{-3} min^{-1}, which is about 3 fold faster than the native one. This activation was observed only when an intercalator was tethered at this specific junction point. Introduction of an intercalator to the catalytic loop or binding arm near the cleavage site significantly lowered the enzymatic activity (See ESI Fig. 2†).

In order to raise the catalytic activity, various intercalators were tethered at this junction point. We found that catalytic activity was improved as long as an intercalator was introduced *via* D-threoninol directly through amide bond (Fig. 3).‡ Among them, anthraquinone (t-Anth) activated the DNAzyme most efficiently: its k_{obs} (17.4×10^{-3} min^{-1}) is about 8 times larger than that of the native one.[11] In contrast, insertion of methylene between the intercalator and amide bond significantly lowered the catalytic activity. For example, direct introduction of a pyrene *via* threoninol through amide bond (X = t-Pyr) accelerated the RNA scission ($k_{obs} = 7.5 \times 10^{-3}$ min^{-1}). But when methylene was inserted between the pyrene and amide bond (X = t-CH_2-Pyr), the enzymatic activity became much lower: k_{obs} (1.1×10^{-3} min^{-1}), which is only 1/7 of the t-Pyr, even lower than the native one. Similarly, introduction of an azobenzene to this junction point on (*S*)-3-amino-1,2-propanediol (pro-Azo), which involves a methylene between the amide bond and main chain, also lowered the enzymatic activity. These results indicate that the intercalator should be directly tethered to the main chain for the activation.

Interestingly, enzymatic activity of t-Azo-tethered DNAzyme was significantly lowered by the *trans* → *cis* photo-isomerization of the azobenzene moiety by UV light irradiation (compare

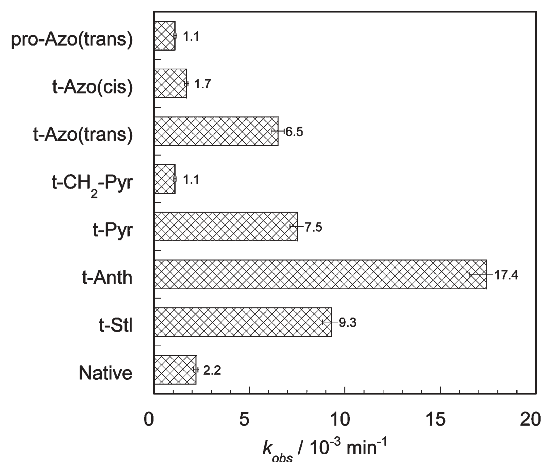

Fig. 3 Pseudo-first order rate constants of various DNAzymes modified with intercalators listed in Fig. 1 under single turn over conditions at 37 °C in the presence of 1.0 M NaCl and 10 mM $MgCl_2$ at pH 7.5 (50 mM Tris buffer). Concentrations of DNAzyme and substrate RNA were 10 μM and 0.8 μM, respectively.

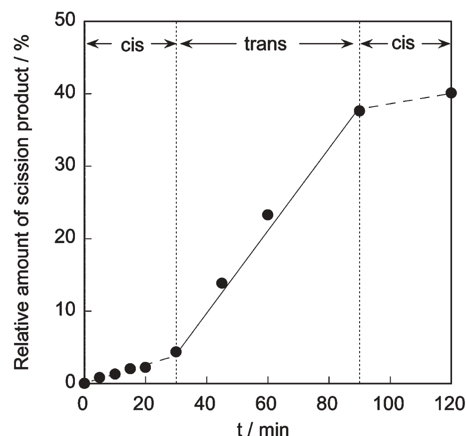

Fig. 4 Photoregulation of RNA cleavage by t-Azo-tethered DNAzyme by irradiating with UV or visible light. Before starting the reaction, UV light was irradiated for 15 min. After 30 min of incubation, visible light was irradiated for 2 min.[12] Finally, UV light was again irradiated at 90 min for 2 min.

t-Azo(*trans*) with t-Azo(*cis*) in Fig. 3).[12] The k_{obs} of the *cis*-form was even smaller than that of the native DNAzyme. Furthermore, this large difference in the activity enabled the reversible photoregulation of DNAzyme reaction.[13] As shown by Fig. 4, RNA cleavage was first suppressed by UV irradiation. But irradiation of visible light accelerated the cleavage reaction because the azobenzene was isomerized to the *trans*-form.[14] Again, enzymatic cleavage was suppressed by UV light irradiation.[15] These facts demonstrate that planar structure is essential for the activation of DNAzyme.[16] Consistently, all the planar molecules used in the present study raised the cleavage rate compared with the native one.

In conclusion, covalent introduction of an intercalator into DNAzyme at the boundary of the catalytic loop and binding arm efficiently activated the RNA scission. By the introduction of an azobenzene at this point, photoregulation of DNAzyme was also possible.

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Notes and references

‡ See Supplementary Information for syntheses of phosphoramidite monomers. All the modified DNAs listed in Fig. 1 were purified by reversed-phase HPLC and characterized by MALDI-TOF MS. MALDI-TOF MS for the DNAzyme tethering t-Azo: obs. 9880.0 (calc. for [t-Azo + H^+]: 9881.8), t-Pyr: obs. 9898.5 (calc. for [t-Pyr + H^+]: 9901.8), t-Anth: obs. 9905.2 (calc. for [t-Anth + H^+]: 9907.8),t-Stl: obs. 9876.0 (calc. for [t-Stl + H^+]: 9880.9), t-CH_2–Pyr: obs. 9912.2 (calc. for [t-CH_2–Pyr + H+]: 9915.9), pro-Azo: obs. 9864.2 (calc. for [pro-Azo + H^+]: 9867.8).

§ Typical procedure of RNA scission is as follows: first, a mixture of substrate RNA and DNA enzyme were annealed in buffer solution (Tris-HCl buffer (pH 7.5) involving NaCl) by heating at 80 °C for 30 s and cooling to 37 °C for 15 min. After this annealing procedure, $MgCl_2$ was added to start the scission and the reaction mixture was incubated at 37 °C. Final concentrations of DNA enzyme, substrate RNA, and Mg^{2+} were 10 μM, 0.8 μM, and 10 mM, respectively, in 50 mM Tris-HCl (pH 7.5) and 1.0 M of NaCl as buffer. During the reaction, 4 μL of the reaction mixture

was sampled and added to 4 μL of loading buffer (50 mM EDTA, 45 mM Tris-HCl, 45 mM borate, 7.0 M urea) to terminate the reaction. Finally, 8.0 μL of the resulting mixture was subjected to electrophoresis on a 20% polyacrylamide gel containing 7.0 M urea. Imaging and quantification of the digested RNA was carried out on a Fuji film FLA-3000 G fluorescent analyzer.

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12 From 15 min before adding $MgCl_2$, UV light from a 6 W UV-A fluorescent lamp (FL6BL-A, Toshiba) was started, to irradiate the sample solution through a UV-D36C filter (from Asahi Technoglass). Throughout the reaction UV light was continuously irradiated to avoid thermal *cis* → *trans* isomerization. The intensity of the UV light was below 100 $\mu J\ s^{-1}\ cm^{-2}$.
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