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Efficacy of Base-Modification on Target Binding of Small Molecule DNA Aptamers

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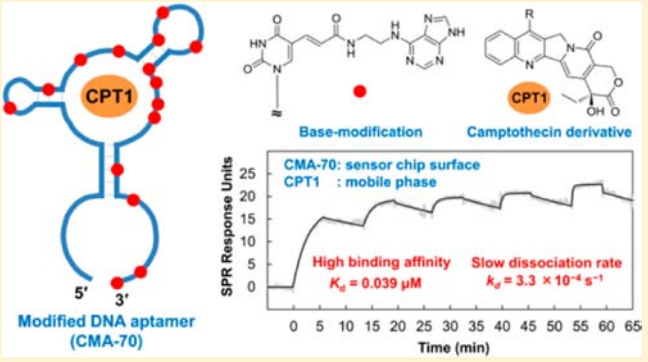
Supporting Information

ABSTRACT: Nucleic acid aptamers are receptors of single-stranded oligonucleotides that specifically bind to their targets. Significant interest is currently focused on development of small molecule aptamers owing to their applications in biosensing, diagnostics, and therapeutics involving low molecular weight biomarkers and drugs. Despite great potential for their diverse applications, relatively few aptamers that bind to small molecules have been reported, and methodologies to enhance and broaden their functions by expanding chemical repertories have barely been examined. Here we describe construction of a modified DNA library that includes (*E*)-5-(2-(*N*-(2-(*N*^{6}-adeninyl)ethyl))carbamylvinyl)-uracil bases and discovery of high-affinity camptothecin-binding DNA aptamers using a systematic evolution of ligands by the exponential enrichment method. Our results are the first to demonstrate the superior efficacy of base modification on affinity enhancement and the usefulness of unnatural nucleic acid libraries for development of small molecule aptamers.



INTRODUCTION

Various modifications of DNA/RNA base, sugar, and phosphate have been proposed to improve performance of nucleic acid aptamers.[1] Among these, some modifications of the sugar and phosphate moieties have led to enhanced nuclease resistance.[2] This improved biostability may be sufficient to allow use of nucleic acid aptamers as therapeutic drugs. Some of these modifications have successfully stabilized the active aptamer conformations, resulting in increased target-binding affinity.[3] Efficacy of these base modifications has recently been demonstrated by Vaught et al.[4] and Gold et al.[5] They showed that modified DNA aptamers containing C5-modified-2′-deoxyuridine with a tryptophan side chain can bind to so-called "difficult protein targets" for which standard DNA/RNA SELEX does not furnish high-affinity aptamers. In contrast, efficacy of base modifications for acquisition of high-affinity receptors for small molecule targets still remained unproved. Indeed, extremely few examples of base-modified DNA/RNA aptamers that recognize a small molecule have been published to date.[6]

The first example[7] was presented by Battersby et al. in 1999. They described a modified DNA aptamer containing a 5-(3-aminopropyl)-uracil base that forms a 1:2 complex with adenosine-5′-triphosphate (ATP). The DNA aptamer with thymine replacing the modified uracil retained the ability to bind ATP, although the affinity for ATP was lowered, indicating that introduction of the foreign functionality did not significantly influence the activities of the selected aptamer. Another example[8] reported by Vaish et al. described an ATP-binding RNA aptamer in which 3-aminopropyl groups were introduced at the C5 position of the uracil base. This aptamer requires the introduced functionality for its ATP-binding activity and interacts with the α- and β-phosphates of ATP. However, an ATP-binding natural RNA aptamer can also interact with the α- and β-phosphates via coordination of a divalent Mg^{2+} cation and can clearly distinguish ATP, ADP, and AMP. In addition, the ATP-binding affinity of the modified RNA aptamer is almost the same as those of the natural DNA/RNA aptamers reported to date;[6b] their apparent dissociation constants (K_ds) are around the low micromolar range in normal saline.

Among the other examples,[9] we demonstrated a unique binding property caused by base modification.[9c] A modified DNA aptamer that included 5-(6-aminohexyl)-carbamoylmethyl-uracil bases specifically bound to the (*R*)-isomer of a thalidomide derivative, demonstrating that modified aptamers could exhibit high enantioselectivity for a highly symmetric, low molecular weight target. However, we could not prove that the base modification enhanced binding affinity, because the

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corresponding natural DNA aptamers could not, unfortunately, be recovered from DNA pools in our experiments. Nevertheless, base modification still attracts many researchers because far greater numbers of base-modified 2′-deoxynucleoside triphosphates (dNTPs) are available as substrates for polymerase reactions compared with the other types of modification.[10] Particularly, substitutions at the C5 position of pyrimidine dNTPs and at the C7 position of 7-deazapurine dNTPs are well tolerated by DNA polymerases. Since polymerase reactions involving modified nucleoside triphosphates are inevitable processes in SELEX experiments using unnatural nucleic acid libraries, this knowledge indicates great potential and expandability of base modification, of DNA in particular, to create a novel class of small molecule aptamers.

In this study, two independent SELEX experiments were conducted targeting the camptothecin derivative 1 (CPT1), one using a natural DNA library and the other using a base-modified DNA library (Figure 1). We introduced *N*-(2-(*N*^{6}-adeninyl)ethyl))carbamylvinyl group at the C5-position of the uracil base as a foreign functionality, expecting that it would increase the probability of inter- and intramolecular hydrogen-bond formation and stacking interactions (Figure S1), and for providing various cavities that can accommodate whole or partial structures of target molecules.

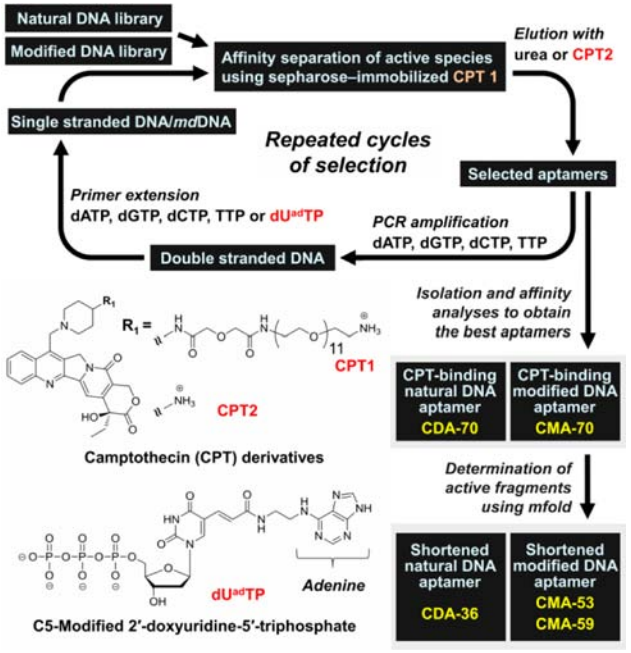

Figure 1. Schematic illustration of CPT-binding aptamer selection from natural/modified DNA libraries using SELEX and chemical structures of key compounds (CPT1, CPT2, and dU^{ad}TP).

EXPERIMENTAL SECTION

Target and Reference Compounds. Chemical structures of all analytes used for affinity analyses are presented in Figures 1 and S2A. (*S*)-(+)-Camptothecin (CPT) and yohimbine hydrochloride (Yh) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Riboflavin-5′-phosphate sodium (FMN) and guanosine-5′-triphosphate (GTP) were purchased from Yamasa Corporation (Chiba, Japan) and Roche Diagnostics K.K. (Tokyo, Japan), respectively. The seven-substituted camptothecin derivatives CPT1 and CPT2 were synthesized from CPT[11] (see Supporting Information).

Polymerase. KOD Dash DNA polymerase (Toyobo Co. Ltd., Osaka, Japan)[12] was used as a catalyst for enzymatic syntheses of libraries, aptamers, and their variants.

Enzymatic Preparations. The modified DNA aptamers (CMA-70, CMA-53, and CMA-59) were enzymatically synthesized using appropriate primers, templates, and substrate triphosphates (dATP, dGTP, dCTP, and dU^{ad}TP). A chemical variant of CMA-59, CMA-59E, was prepared using dU^{et}TP instead of dU^{ad}TP. Chemical structures of dU^{ad}TP and dU^{et}TP are presented in Figures 1 and S2B. These were synthesized from (*E*)-5-(2-carboxyvinyl)-2′-deoxyuridine (see Supporting Information).

SELEX Experiments. Classical SELEX experiments[13] using affinity gels for positive and negative selections were performed to acquire CPT-binding aptamers from natural and modified DNA pools (libraries A and B), respectively. For positive and negative selections, two affinity gels with amide bond-immobilized CPT1 and 2-aminoethanol were prepared, respectively. Both libraries comprised 5′-(6-carboxyfluorescein)-labeled 70-mer oligodeoxyribonucleotides (ODNs), with 30-mer random regions of A, G, C, and T for library A or (*E*)-5-(2-(*N*-(2-(*N*^{6}-adeninyl)ethyl))carbamylvinyl)-uracil instead of T for library B, flanked by constant 20-mer forward and reverse primer-binding sequences. After gel affinity separations, active species were amplified by polymerase chain reaction (PCR) with four natural 2′-deoxynucleoside-5′-triphosphates (dNTPs) to give the corresponding double-stranded ODN (dsODN). Subsequently, in the selection of library A, the antisense strand of 5′-monophospate-labeled ODNs was selectively degraded by λ-exonuclease treatment. The resulting single-stranded ODN (sense strand) was purified by polyacrylamide gel electrophoresis (PAGE) and used as a library for the next round. To select ODNs for library B, one primer PCR was performed using the forward primer, substrate triphosphates dATP, dGTP, dCTP, and dU^{ad}TP, and the PCR-amplified ODN as a template. As sense strands migrated much more slowly than the template, the single-stranded modified ODN for the next round was retrieved from the reaction mixture by PAGE purification. In total, 11 rounds were performed to select ODNs for library A, while 9 rounds were performed to select those for library B. Active species were eluted from the positive gel using a buffer containing 7 M urea until the fifth and seventh rounds for library A and library B, respectively, or 0.2 mM CPT2 for the remaining rounds. After confirming saturation of active species enrichment, aptamers were isolated by a cloning method, and 15 aptamers were recovered from each enriched library (A and B). Detailed protocols for these experiments are described in Supporting Information.

Surface Plasmon Resonance (SPR) Analyses. Affinity profiles of aptamers were characterized by SPR spectroscopy using Biacore 3000 and Biacore X (GE Healthcare Japan; Tokyo, Japan) (Figures 2–4 and S4–6). Each aptamer was biotinylated at the 5′ end and was immobilized on a streptavidin-coated chip (sensor chip SA). Appropriate buffer solutions containing target and reference compounds (analytes) were injected over the sensor surface. Their K_d values and association and dissociation rate constants (k_a and k_d, respectively) were determined using either single-cycle or classical multiple-cycle kinetic methods[14] with the assumption that immobilized aptamers bind analytes in a 1:1 ratio. Detailed procedures of sensor chip preparations and analytical conditions are described in Supporting Information.

Affinity Analyses Using Solution-Based Methods. Affinity profiles for a shortened CPT-binding modified DNA aptamer (CMA-59) and those for 35-mer FMN-binding RNA aptamer (FRA)[15] and 41-mer GTP-binding RNA aptamer (GRA),[16] which are known to be high affinity binding receptors, were also characterized by solution-based methods (Figures 5 and S7 and S8) because SPR-based equilibrium constants do not necessarily match those obtained from solution-based methods.[17] Because CPT1 and FMN were fluorescent molecules, CMA-59 and FRA were analyzed by fluorescence titrations using an LS-55 spectrofluorophotometer (Perkin-Elmer Japan Co., Ltd., Kanagawa, Japan). GTP was nonfluorescent, so GRA affinity was determined by isothermal titration calorimetry (ITC) using a MicroCal iTC200 system (GE Healthcare Japan, Tokyo, Japan).

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Detailed experimental conditions are described in Supporting Information.

Conformational Validation. Validity of the predicted secondary structure of CMA-59 (Figure 2) was further assessed to determine whether strand displacement at the putative stem part of CMA-59 affected its target binding activity. Its corresponding ODN (cODN-17; 5′-GTC GCA CGC CAG ACA CG-3′), which included the first 17 bases from the 5′-end of CMA-59 and comprised only natural nucleotides, could be hybridized with the last eight bases (5′-CG**t** GCG AC-3′; **t** = (*E*)-5-(2-(*N*-(2-(*N*^{6}-adeninyl)ethyl))carbamylvinyl)-uracil) from the 3′-end of CMA-59 to form an 8-bp duplex with two dangling strands. The cODN-17 concentration dependence of CMA-59 binding affinity for CPT1 was verified by measuring changes in fluorescence polarization using the LS-55 (Figure 6). Detailed experimental conditions are described in Supporting Information.

RESULTS

Sequence Analyses. From each library, 15 sequences were selected (Tables S2 and S3). Three-tiered G-quadruplex motifs $G_3NG_3NG_3N_nG_3$ (n = 1−2 and N = A, G, C, or T) were found in all 15 natural DNA aptamers. This may be plausible because G-quadruplex motifs are often seen in small molecule DNA aptamers.[18] In contrast, the obtained modified DNA aptamers contained either G_3**t**G_2**t** or GA_3G_2**t** motifs but no G-quadruplex motifs.

Affinity Analyses. The best CPT-binding natural DNA aptamer was identified from the selected sequences through affinity analyses using fluorescence titration (Table S4 and Figure S3) and was named CDA-70 (70 mer). Subsequently, a shortened natural DNA aptamer CDA-36 (36 mer) with the highest binding affinity was identified through analyses of sequence conservation and sequence−activity correlations and structural predictions using Zuker mfold[19] (Figure 2). Meanwhile, the best CPT-binding modified DNA aptamer was identified from the selected sequences through affinity analyses using SPR spectroscopy (Table S5) and was named CMA-70 (70 mer). Although mfold regards input sequences as fully natural nucleotides, we compulsorily applied mfold to secondary structure predictions of the modified DNA aptamer CMA-70. Stable structures 1 and 2 were predicted by mfold at 25 °C in the presence of 138 mM Na^+ and 2.5 mM Mg^{2+}. Further, shortened modified aptamers CMA-53 (53 mer) and CMA-59 (59 mer) were designed and prepared on the basis of structures 1 and 2, respectively (Figure 2). Compared with full-length CMA-70, CMA-59 substantially retained target-binding affinity but CMA-53 did not (Table 1 and Figure 3A−C), indicating that CMA-70 would form conformations that are similar to structure 2 and not structure 1. CMA-70 and CMA-59 showed superior binding affinities to CPT1 with K_d values of 0.039 and 0.086 μM, which are 28- and 13-fold lower than that of CDA-36 (K_d = 1.1 μM), respectively. Under the same conditions as those used for SPR experiments, the 35-mer FRA and the 41-mer GRA bound to their targets with K_d values of 0.74 and 12 μM, respectively. The small K_d value for CMA-59 binding to CPT1 was also confirmed by both fluorescence titration and fluorescence polarization measurements: K_d = 0.071 ± 0.012 and 0.081 ± 0.015 μM, respectively (Figure 5). Likewise, the K_d values for FRA obtained from fluorescence titration and that for GRA from ITC were determined to be, respectively, 0.78 ± 0.04 and 9.8 ± 0.9 μM (Figures S7 and S8); these were close to those from SPR analyses (Table 1).

Specificity Analyses. As shown in Table 1, CMA-70 and CMA-59 demonstrated not only high binding affinity but also high binding specificity; they exhibited at least 1700-fold weaker binding affinities to nontargeted azaheterocyclic compounds, such as FMN and Yh (Figure S5A). In contrast, CDA-36 distinguished CPT1 from FMN and Yh with only 27- and 45-fold differences in binding affinity, respectively. FRA can

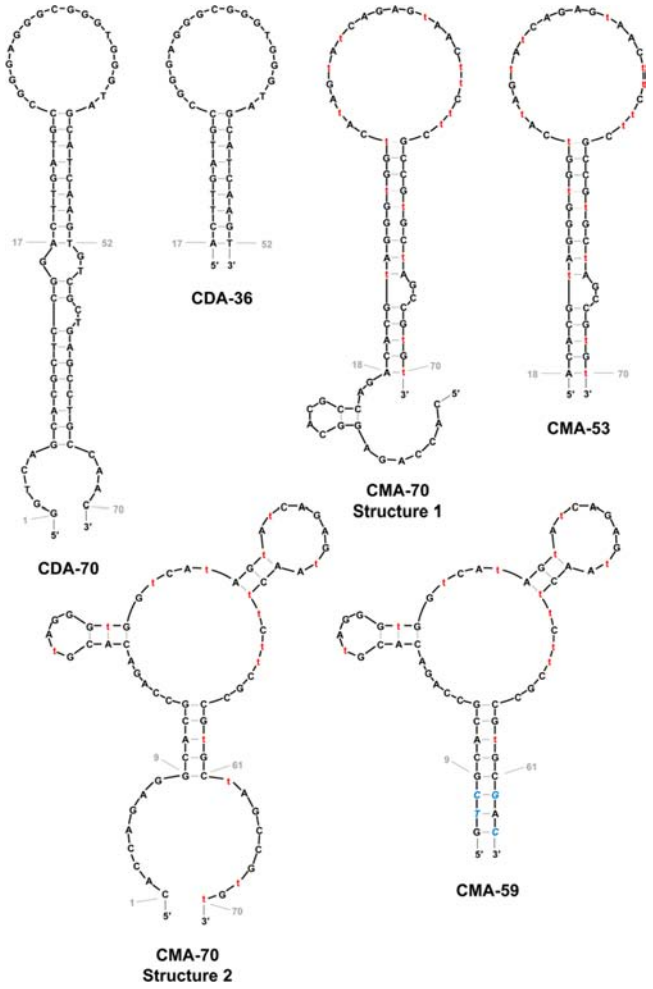

Figure 2. Predicted secondary structures of aptamers and fragments using mfold−DNA folding form; (*E*)-5-(2-(*N*-(2-(*N*^{6}-adeninyl)-ethyl))carbamylvinyl)-uracil and bases introduced to form stem structures are shown in letters (t) and italic capitals, respectively.

Table 1. Comparison of Binding Affinity and Specificity of CPT-Binding Aptamers with Those of the Reference FRA and GRA

	K_d (μM)[b]				
	analytes				
aptamer[a]	CPT1	CPT	FMN	GTP	Yh
CDA-36	1.1	n.d.	30	n.d.	50
CMA-70	0.039	n.d.	67	NB	NB
CMA-53	0.79	n.d.	n.d.	n.d.	n.d.
CMA-59	0.086	0.68	170	NB	NB
CMA-59N	130	n.d.	NB	n.d.	n.d.
CMA-59E	150	n.d.	NB	n.d.	n.d.
FRA	NB	n.d.	0.74	NB	NB
GRA	330	n.d.	160	12	NB

[a]Sequences of aptamers are presented in Table S6. [b]K_d values were determined using SPR-based technology (Biacore 3000). NB stands for no binding, and n.d. indicates that the K_d values were not determined.

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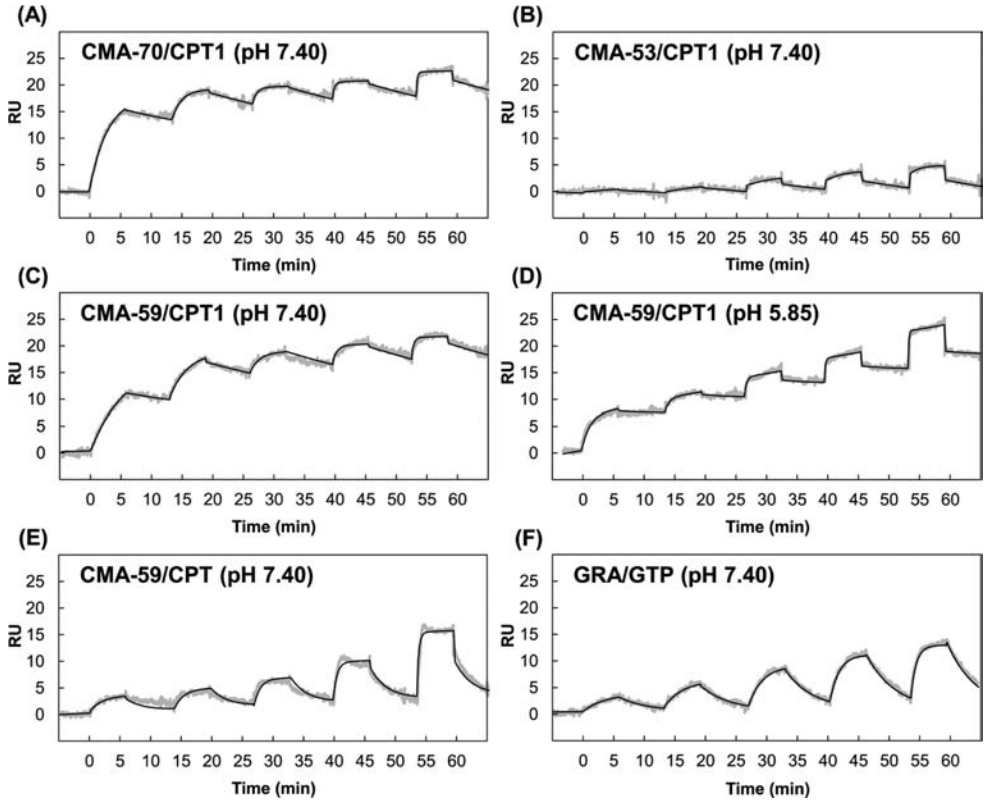

Figure 3. SPR sensorgrams of the interaction between aptamers and their targets. Measurements were performed using Biacore 3000 with single-cycle kinetics. Gray and black lines indicate observed sensorgrams and the fit of these responses to a 1:1 kinetic titration model.

strictly discriminate its target (i.e., FMN) from nontargets (i.e., CPT1, GTP, and Yh). However, such superior specificities, comparable to those of CMA-70, CMA-59, and FRA, were not seen in SPR analysis of GRA (Figure S5B,C). Interestingly, CMA-59 distinguished CPT1 from CPT with a difference of 7.9-fold in binding affinity, indicating that the substituent at the seventh position of CPT1 was recognized by this aptamer (Figure 3C,E).

Kinetics Analyses. As shown in Table 2, a noteworthy feature of CMA-70 and CMA-59 in target binding is their very slow dissociation rates (k_d = 0.00033 and 0.00037 s^{-1}), which are at least 270-fold lower than that of CDA-36 (k_d > 0.1 s^{-1}). Such properties of CMA-70 and CMA-59 were observed during the dissociation from CPT1 and CPT but not from nontargets (Figures 3A,C,E and S5A). Under the same conditions as that used in SPR experiments, GRA, which is well characterized as a slow off-rate aptamer, also exhibited a slow dissociation rate constant (k_d = 0.0035 s^{-1}) for the target GTP (Figures 3F and S4E). Thus, the k_d values of CMA-70 and CMA-59 were found to be 11- and 9.5-fold lower than that of GRA, respectively. In contrast, CDA-36 and FRA showed rapid association and dissociation with their respective targets (Figures S4A,D and S6). This binding property has also been observed in small molecule aptamers, such as ATP-binding aptamer[20] and arginine-binding aptamer.[21] Finally, pH dependency of the slow off-rate property was assessed (Figure 3D); the k_d of CMA-59 for CPT1 increased 2.1-fold when the pH decreased from 7.40 to 5.85, indicating that it may be influenced by acidic conditions found in cancer cells.[22]

Table 2. Apparent Association and Dissociation Constants Determined Using Biacore 3000[a]

aptamer	analytes	k_a ($M^{-1}\cdot s^{-1}$)	k_d (s^{-1})	χ^2 (RU)^2
CDA-36	CPT1	$>10^5$	$>10^{-1}$	NA
CMA-70	CPT1	8500	0.00033	0.14
CMA-53	CPT1	810	0.00065	0.26
CMA-59	CPT1	4200[b]	0.00037[b]	0.27[b]
CMA-59	CPT1	18 000[c]	0.00076[c]	0.19[c]
CMA-59	CPT	8500	0.0058	0.94
FRA	FMN	41 000	0.030	0.56
GRA	GTP	300	0.0035	0.10

[a]All analyses were conducted using only phosphate buffer at pH 7.40 except for those of CMA-59/CPT1. [b]Performed using phosphate buffer at pH 7.40. [c]Performed using phosphate buffer at pH 5.85.

Effects of Base Modification. To identify which parts of the foreign functionality affect the binding properties, two chemical variants of CMA-59, CMA-59N and CMA-59E, in which all (*E*)-5-(2-(*N*-(2-(N^6-adeninyl)ethyl))carbamylvinyl)-uracil bases were replaced with natural thymine and (*E*)-5-(2-(*N*-ethyl)carbamylvinyl)-uracil bases (Table S6), respectively, were prepared and analyzed using SPR. Both CMA-59N and CMA-59E showed highly inferior binding affinities to CPT1 that were at least 1500-fold weaker than that of CMA-59, although they still retained specificity; they did not bind to FMN (Table 1). Furthermore, the slow off-rate property was not observed in the target binding of CMA-59N and CMA-59E (Figure S4B,C). These results demonstrated that the adenine moiety but not the linker moiety of the foreign functionality critically contributes the strong target binding and slow off-rate properties observed. This indicates that, in concert with regular nucleobases on furanose rings, the introduced adenine bases could provide unique spatial arrangement and orientation of

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nucleobases to form a highly stable complex with the target molecule.

Mg^{2+} Dependency. Divalent metal-ion dependency of base-modified DNA aptamers for small molecules in target binding has not yet been studied precisely. In general, small molecule RNA aptamers exhibit considerable Mg^{2+} dependency in target binding.[20c] Indeed, as shown in Figure 4, binding

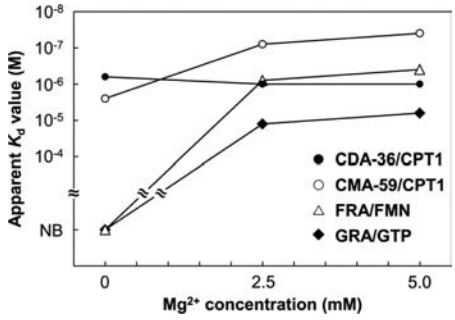

Figure 4. Mg^{2+} dependencies of CDA-36, CMA-59, FRA, and GRA on their binding affinities. NB stands for no binding. Measurements were made using Biacore 3000.

activities of the RNA aptamers FRA and GRA were completely lost in the absence of Mg^{2+} ions and were markedly recovered when the solution contained millimolar concentrations of Mg^{2+}. In contrast, the binding activity of natural DNA aptamer CDA-36 with a three-tiered G-quadruplex motif was hardly affected by Mg^{2+}. This may be because the G-quadruplex could form a stable core structure without Mg^{2+} and maintain the active form of CDA-36. Interestingly, modified DNA aptamer CMA-59 showed considerable Mg^{2+} dependency; its binding activity was retained in the absence of Mg^{2+} but increased by 28- and 56-fold as Mg^{2+} concentration was raised to 2.5 mM and 5.0 mM, respectively. Hence, Mg^{2+} ions may not be essential, but may mediate aptamer−target interactions and play an important role in the formation of target-binding sites of best fit.

Validity of Predicted Conformation. Fluorescence polarization results for CPT1 with equimolar amounts of CMA-59 decreased as the cODN-17 concentration increased, whereas the results for those without CMA-59 remained nearly unchanged for the same cODN-17 concentration range (Figure 6A). Thus, cODN-17 did not compete with CMA-59 for binding to CPT1 but did reduce the target binding activity of CMA-59. These results strongly indicate that excess amounts of cODN-17 cause strand displacement in the 8-bp stem of CMA-59 to eventually render CMA-59 inactive (Figure 6B) and demonstrate the validity of the predicted secondary structure of CMA-59, which was designed from CMA-70 (structure 2) as a candidate active form (Figure 2).

■ DISCUSSION

Although various methods are available to measure binding affinities of small molecule aptamers, SPR is one of the most common methods for determining K_d values and has an advantage that it can also provide k_a and k_d values. However, these values can vary depending on methods and conditions used. Accordingly, we initially sought high affinity aptamers that are suitable as a standard reference and chose the 35-mer FRA,[15] which is one of the best characterized aptamers with submicromolar affinity to the target FMN. Subsequently, we confirmed that its K_d value, which was determined using

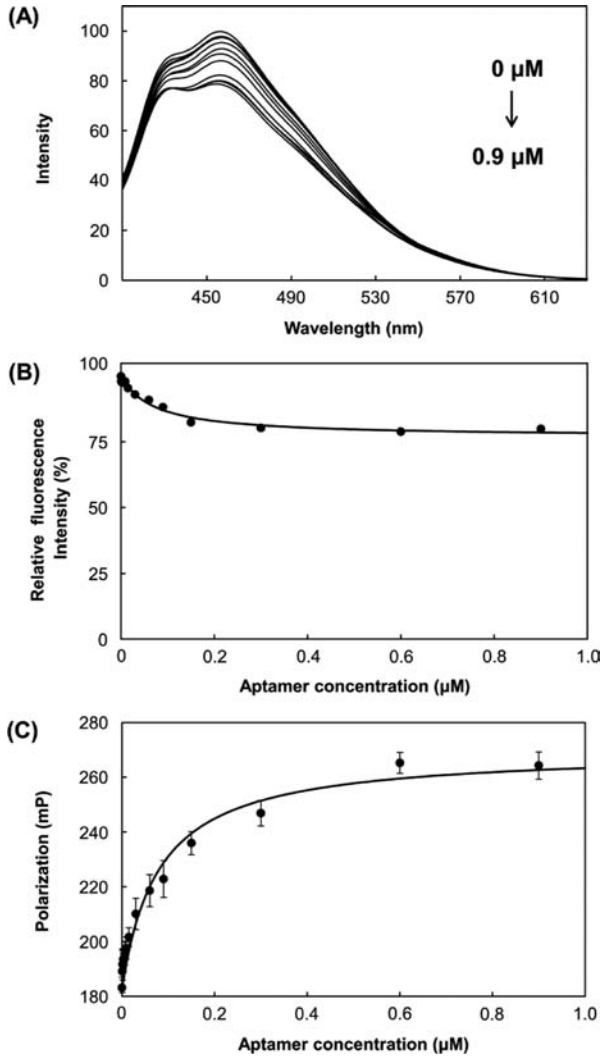

Figure 5. Fluorescence titration of target CPT1 with increasing concentrations of aptamer CMA-59. (A) Fluorescence spectra for CPT1 with varying CMA-59 concentrations and excitation at 372 nm. (B) Titration curve for relative fluorescence intensity at 456 nm versus aptamer concentration; CPT1 fluorescence without CMA-59 was set at 100%. (C) Titration curve for CPT1 polarization versus CMA-59 concentration; polarization was monitored at 456 nm using an excitation wavelength of 372 nm.

analytical affinity chromatography in the literature (K_d = 0.5 μM in the presence of 250 mM Na^+ and 5 mM Mg^{2+} at pH 7.6),[15] was in good agreement with that obtained using SPR analyses (K_d = 0.40 μM in the presence of 138 mM Na^+, 2.7 mM K^+, and 5 mM Mg^{2+} at pH 7.40). Hence all SPR analyses were performed according to the procedures and conditions used for the reference FRA. Although the dissociation constant of the 41-mer GRA for GTP (K_d = 12 μM) was far greater than the reported value (K_d = 0.075 μM), the slow off-rate property of GRA could confirm with the dissociation rate constant (k_d = 0.0035 s^{-1}) relatively close to the reported value (k_d = 0.001 s^{-1}: provided, the k_d of 0.06 min^{-1} was given in the literature).[16a] The large difference in the K_d values, i.e., the k_a values, in this case may be due to SPR conditions used. In the previous study,[16a] target compounds were immobilized on sensor chips, whereas aptamers were immobilized[23] in our experiments, implying that the different k_a values may have been brought about by accessibility of mobile molecules

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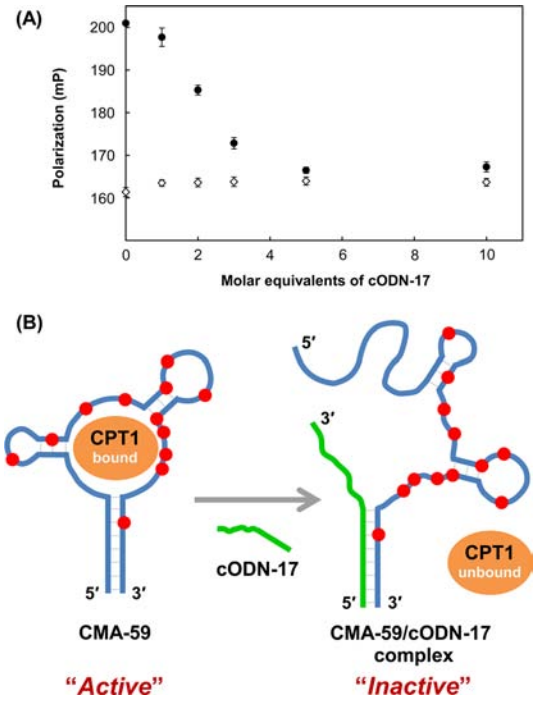

Figure 6. Inactivation of CMA-59 during target binding with increased concentrations of cODN-17. (A) Fluorescence polarization results for CPT1 (0.6 μM) with equimolar CMA-59 and with different cODN-17 concentrations (0–6 μM) (closed circles) and those for CPT1 (0.6 μM) without CMA-59 and with different cODN-17 concentrations (0–6 μM) (open diamonds); polarization was monitored at 456 nm using an excitation wavelength of 372 nm. (B) Illustration of the conformational change due to cODN-17. Red closed circles represent nucleotides with the base modification.

(analytes) to counterpart molecules immobilized on SPR sensor surfaces. In addition, the buffer used in the literature (200 mM K^+ and 5 mM Mg^{2+} at pH 6.2) is different from that used in this study. The higher salt concentration and lower pH may stabilize the predocking conformation of GRA to smoothly accommodate GTP, which raises the k_a value in the target binding of GRA. Although there is discordance in the binding affinity, the K_d value determined by SPR under the present conditions, approximately matched with that obtained by ITC.

Thus, the obtained K_d and k_d values of CPT-binding natural/modified DNA aptamers and their variants were enough to compare with each other and thus enable determination of the enhanced effects of base modification on the binding properties of small molecule aptamers.

To identify the active form of CMA-70, the shortened aptamers CMA-53 and CMA-59 were prepared on the basis of secondary structural predictions using mfold. This trial is challenging because methodologies for predicting modified nucleic acid conformations have not yet been established and because little is known on the acceptability of existing methods for chemical modifications. In our previous study,[9c] we successfully identified an active fragment of a thalidomide-binding modified DNA aptamer from three candidates, the structure of which was proposed on the basis of a three-way junction model predicted using GENETYX software. In the current study, prior to CMA-70 analysis, the structure of CDA-70 was predicted using mfold, and the 36-mer shortened aptamer CDA-36 capable of forming a stem-loop structure (Figure 2) was generated. This CDA-36 structure contains a G-quadruplex motif in the loop that is connected to a flanking duplex stem, which can also been seen in previously reported DNA aptamers for ATP[20b] and thrombin.[24] The target-binding affinity of CDA-36 was observed to be comparable with that of CDA-70, indicating that the mfold prediction in this case was acceptable. With regard to the modified DNA aptamer, although CMA-53 and CMA-59 share a successive 44-mer sequence, only the latter retained the original binding activity, indicating that structure 2 is the active form of CMA-70. Furthermore, cODN-17 markedly inactivated CMA-59, indicating that CMA-59 cannot form an adaptor to fit the target without being tied with the 8-bp stem, while the stem itself is not the target binding site (Figure 6). These results implied that the mfold prediction is appropriate for modified DNA aptamers in this case. Therefore, we expect the present study to form the basis for exploring valid computer simulations to predict structure–function correlations of chemically modified aptamers.

The modified DNA aptamer CMA-70 possesses immense potential as a drug carrier for drug delivery systems (DDS).[25] Its targets, CPTs, are potent antitumor drugs and are widely available for cancer therapy.[26] DNA is a potential material for DDS, owing to the following drug carrier properties: (i) DNA aptamers with specificity for diverse targets can be created using SELEX methods, (ii) three-dimensional nanostructures of various shapes can be designed and fabricated based on base sequences,[27] (iii) conformations are changeable depending on the *in vivo* environment.[28] Recently, carrier DNAs for the antitumor drugs doxorubicin (DOX)[25a,c] and daunomycin (DAU)[29] have been designed and developed. These exploit intercalation to ensure strong interactions between DNA and drugs. The use of such noncovalent interactions may facilitate drug release. Unlike DOX and DAU, CPTs do not intercalate into DNA but weakly bind to DNA with K_d values of several hundred micromolar or more.[30] Therefore, it has been difficult to create carrier DNAs that tightly bind to CPTs. Nonetheless, CMA-70 and CMA-59 showed high binding affinities and extremely low dissociation rates, and moreover, CMA-59 exhibited pH dependency in target binding, which generate carrier DNAs for nonintercalating CPTs, and indicate that base modification can expand the range of potential target drugs. In future studies, a minimized structure of CMA-70, which can be shortened further than CMA-59, will be conjugated to tumor-cell-binding DNA aptamers,[31] antibodies,[32] and peptide ligands,[33] and subsequently clustered on nanoparticles[34] to construct a novel class of aptamer-based controlled release DDS.

■ CONCLUSION

In conclusion, we conducted SPR analyses of aptamers recovered from natural and modified DNA libraries, which indicated that modified DNA libraries had enhanced potential compared with natural DNA libraries to provide high affinity aptamers with low dissociation rate constants. As per our knowledge, this is the first demonstration of improved binding affinity of small molecule DNA aptamers comprising modified bases. Furthermore, we demonstrated the application of mfold to modified DNA, which suggests that methodologies for structural prediction of modified DNA can be established by improving algorithms on the basis of accumulating experimental data. We believe there is immense potential for chemical modifications in small molecule aptamers, which will

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lead to biomedical and biosensing applications in the near future.

■ ASSOCIATED CONTENT

Supporting Information

Figures, tables, and additional text showing detailed experimental procedures, characterization of all compounds, and additional experimental results. 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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