

Isolation of a fluorophore-specific DNA aptamer with weak redox activity

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Background: *In vitro* selection experiments with pools of random-sequence nucleic acids have been used extensively to isolate molecules capable of binding specific ligands and catalyzing self-modification reactions.

Results: *In vitro* selection from a random pool of single-stranded DNAs has been used to isolate molecules capable of recognizing the fluorophore sulforhodamine B with high affinity. When assayed for the ability to promote an oxidation reaction using the reduced form of a related fluorophore, dihydrotetramethylrosamine, a number of selected clones show low levels of catalytic activity. Chemical modification and site-directed mutagenesis experiments have been used to probe the structural requirements for fluorophore binding. The aptamer recognizes its ligand with relatively high affinity and is also capable of binding related molecules that share extended aromatic rings and negatively charged functional groups.

Conclusions: A guanosine-rich single-stranded DNA is capable of binding fluorophores with relatively high affinity and of weakly promoting a multiple-turnover reaction. A simple motif consisting of a three-tiered G-quartet stacked upon a standard Watson-Crick duplex appears to be responsible for this activity. The corresponding sequence might provide a useful starting point for the evolution of novel, improved deoxyribozymes that generate fluorescent signals by promoting multiple-turnover reactions.

Introduction

In vitro selection from random-sequence pools of nucleic acids has been used to isolate a wide range of ligand-binding molecules (aptamers) and to obtain catalysts that promote reactions well outside the normal range of ribozyme-mediated transformations (e.g. [1–12]). To become a widely applicable technique for producing useful catalysts, efficient strategies for the *in vitro* evolution of novel enzymes must be developed. Most ribozymes isolated using *in vitro* selection so far have been obtained on the basis of their ability to undergo covalent self-modification, a process that effectively tags the active molecules in a pool and allows their preferential amplification. The selective pressure in these types of experiments is for rapid single turnover under conditions in which at least one of the reactants is already associated with the ribozyme. As such, one might expect to isolate molecules that greatly promote the chemical step of a reaction but that do not efficiently bind substrate or release product.

As an alternative approach, Schultz and coworkers [13,14] have applied the transition-state-analog method, originally developed for catalytic antibodies, to random-sequence RNA pools to isolate true ribozymes that promote multiple-turnover reactions. RNAs selected to recognize the appropriate reaction transition-state analog have been

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Key words: DNA aptamer, fluorophore, G-quartet, *in vitro* selection, sulforhodamine B

Received: 10 June 1998
Revisions requested: 7 July 1998
Revisions received: 13 August 1998
Accepted: 2 September 1998

Published: 9 October 1998

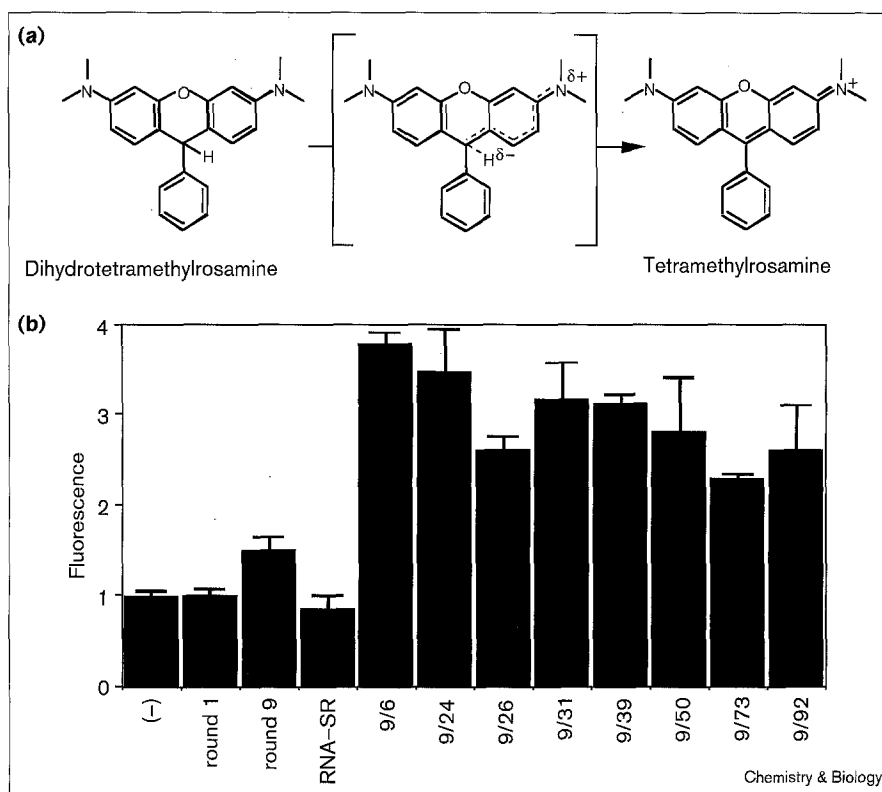
Chemistry & Biology November 1998, 5:609–617
<http://biomednet.com/eleceref/1074552100500609>

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shown to accelerate conformational changes in a biphenyl compound and to catalyze porphyrin metalation. Although this method is successful, catalysts isolated using this technique have failed to match the rate enhancements generally achieved when single-reaction turnover has provided the selective pressure.

We have been interested in developing an alternative strategy for the isolation of multiple-turnover catalysts based on the direct fluorescence-based detection of a ribozyme-generated reaction product. A wide range of fluorogenic substrates are available both commercially and by straightforward synthesis from simple precursors. Hydrolysis, oxidation or dealkylation of these compounds typically leads to the generation of a fluorescent product. Because fluorophores are detectable at exceedingly low concentrations (single molecules have been visualized using optical microscopy [15,16]), it has been possible to measure the activity of a single fluorogenic substrate-utilizing enzyme [17]. As a first step towards the isolation of multiple-turnover DNA-based enzymes, we have initially attempted to isolate small DNAs that simply bind fluorophores with high affinity. Isolation of such DNAs would provide a logical starting point for future experiments aimed at the evolution of efficient catalysts that generate fluorescent signals. Such motifs, by their ability to co-localize with

Figure 1



The conversion of dihydrotetramethylrosamine (DHTMR) to tetramethylrosamine (TMR).

(a) Oxidation of fluorogenic substrate dihydrotetramethylrosamine yields the product TMR, readily detected and quantitated by fluorimetry. (b) DNA from the first round of selection (round 1) and from the final selected pool (round 9), an RNA-sulfurhodamine aptamer pool isolated by *in vitro* selection (RNA-SR), and DNA from individual clones from round 9 were assayed for the ability to convert DHTMR to TMR as described in the Materials and methods section. -, control. Fluorescence units are arbitrary.

readily detectable fluorophores, might also prove useful as simple sequence tags for labeling larger DNAs.

We now report the isolation and characterization of DNA aptamers that specifically recognize sulfurhodamine B, a sulfonated rhodamine derivative. We have screened ~100 initial isolates from this aptamer pool for molecules capable of catalyzing the conversion of dihydrotetramethylrosamine to the fluorescent product tetramethylrosamine. Several clones within the selected pool weakly promote this oxidation reaction. A relatively simple motif, consisting of a three-tiered G-quartet, appears to be likely to account for high affinity sulfurhodamine binding and catalysis.

Results and discussion

Starting with a pool containing 5×10^{14} different random-sequence single-stranded DNA molecules, we used sulfurhodamine agarose affinity chromatography to isolate fluorophore-specific aptamers as described in the Materials and methods section. After eight rounds of selection and amplification, ~20% of the input DNA bound to the sulfurhodamine column and subsequently eluted upon washing with sulfurhodamine-containing buffer. Two additional rounds of selection failed to increase the fraction bound to the column. The complexity of the final selected pool was determined using restriction analysis, as described previously [18]. Approximately 20 major species

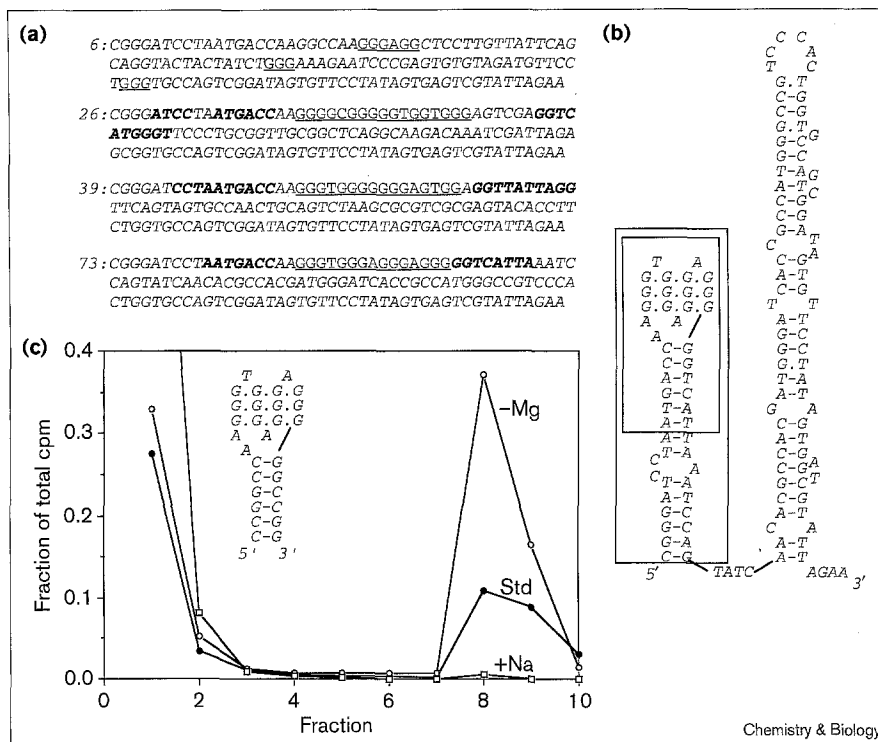
appear in the final pool, together with a large population of minor species.

As an initial test of our strategy to isolate multiple-turnover ribozymes that generate a fluorescent signal, we assayed the selected pool for redox activity with dihydrotetramethylrosamine (DHTMR), a colorless compound that, upon oxidation, yields the fluorescent product tetramethylrosamine (TMR, Figure 1a). The substrate and product of this reaction closely resemble sulfurhodamine and we therefore reasoned that some fraction of molecules within the pool would be able to accommodate substrate binding and possibly stabilize the reaction transition state. Furthermore, the activation barrier for the reaction is relatively low as demonstrated by the tendency for DHTMR to spontaneously oxidize, especially in the presence of light. As such, relatively minor stabilization of the transition state is likely to yield a detectable change in the rate of oxidation.

As shown in Figure 1b, addition of 50 nM DNA from the aptamer pool to DHTMR promotes an increase in its oxidation to TMR by ~60% over that observed with no additive, addition of pre-selected DNA (round 1), or addition of RNA-based sulfurhodamine aptamers isolated in a separate SELEX experiment [19]. Although the activity of the pool is clearly low, the final excess TMR concentration is sufficiently high (~350 nM product after overnight reaction,

Figure 2

(a) The sequences of sulforhodamine-binding DNA aptamers were determined as described in the Materials and methods section. Constant primer sequences are shown in italics, G-rich sequences are underlined, and flanking complementary sequences are shown in bold. (b) Predicted folding of clone 73, generated by the Zuker MFOLD algorithm [20]. Boxed regions correspond to minimal aptamers prepared and tested for binding. Both minimal aptamers bound with essentially identical efficiency to the wild-type DNA. (c) Sulforhodamine agarose binding by a minimal aptamer based on clone 73 with modified helix sequence was assayed under various salt conditions. DNA was applied to the column, washed with seven column volumes of standard or modified buffer, and then eluted with three column volumes of elution buffer (5 mM sulforhodamine in either standard or modified buffer). Std, standard selection buffer (0.1 M KCl, 10 mM Na-HEPES, 5 mM MgCl₂); -Mg, magnesium-free buffer (0.1 M KCl, 10 mM Na-HEPES); +Na, potassium-free buffer (0.1 M NaCl, 10 mM Na-HEPES, 5 mM MgCl₂).



which is approximately seven times the DNA concentration) that it cannot be attributed to single reaction turnover. The activity detected for the entire pool could result from a handful of rare, highly active deoxyribozymes or from the collective activity of many poor catalysts. To distinguish between these possibilities, we tested the activity of individual clones within the final selected pool. Clonally pure single-stranded DNA was obtained by microtiter-plate-based polymerase chain reaction (PCR) amplification of 96 individual transfected colonies. The amplified product was subsequently diluted into a cocktail containing DHTMR and hydrogen peroxide and incubated in the dark overnight. Quantification of the fluorescence signal in each microtiter well suggests that roughly one third of the clones have redox activity. After identifying the most highly reactive clones, purified DNA was prepared for each and assayed as before for the mixed pool. As shown in Figure 1b, the activities of these clones are roughly twice that of the pool as a whole, and their rate enhancement over background oxidation in the best case (9/6) is only 3.7-fold.

To understand how these clones fold and function, with the ultimate goal of engineering better ribozymes or DNAzymes, we selected four of the clones (9/6, 9/26, 9/39 and 9/73) for characterization. Figure 2a shows the sequences determined for each clone. The only obvious conserved motif among the sequences is a short guanosine-rich region characterized by four runs of approximately three guanosines each and flanked by complementary sequences.

To determine whether this motif alone was responsible for binding, we prepared progressively shortened fragments of the clone 73 sequence and measured the ability of the fragments to bind to sulforhodamine agarose (Figure 2c, Table 1). Analysis using the MFOLD program [20] suggested that this sequence folded into two long imperfect helices with the guanosine-rich sequence lying in the loop terminating the 5' helix (Figure 2b). A 50-nucleotide fragment corresponding to the predicted 5'-helical region bound as efficiently as the original aptamer. A 29-nucleotide fragment corresponding to the guanosine-rich region and a flanking helix of just six nucleotides was also found to bind efficiently (Figure 2c). Replacement of the original helical region by a different helix sequence had a minimal effect on activity, suggesting that the helix acts simply to tether the ends of the guanosine-rich loop together rather than directly participating in binding (Table 1). Binding by the minimal binder to sulforhodamine agarose requires potassium and is improved in the absence of magnesium (Figure 2c). The effects of monovalent and divalent cations on binding efficiency are paralleled by similar effects on DHTMR turnover. The omission of magnesium from the oxidation reaction increases the enzymatic activity of the minimal binder by 165%, whereas increasing the potassium concentration from 0.1 M to 0.5 M KCl increases activity to 140%.

Guanosine-rich sequences are known to form stable intermolecular complexes with other guanosine-rich sequences

Table 1

Binding of mutant oligosaccharides to sulforhodamine agarose.

Construct	Sequence	Relative binding
DSR9-73, hairpin 1	CGGGATCCTAATGACCAAGGGTGGGAGGGAGGGGGTCATTAATCCAG	148%
DSR9-73, minimal	ATGACCAAGGGTGGGAGGGAGGGGGTCAT	115%
Substituted helix*	CCGGCCAAGGGTGGGAGGGAGGGGGCCGG	100%
Four-tier quartet	CCGGCCAAGGGGTGGGAGGGAGGGGGGGCCGG	40%
Two-tier quartet	CCGGCCAAAGGTGGGAAGGAAGGGGCCGG	0%
1st G-run, bottom tier	CCGGCCAAAGGTGGGAGGGAGGGGGGCCGG	31%
2nd G-run, bottom tier	CCGGCCAAGGGTGGAAAGGGAGGGGGGCCGG	7%
3rd G-run, bottom tier	CCGGCCAAGGGTGGGAAGGGAGGGGGGCCGG	7%
4th G-run, bottom tier	CCGGCCAAGGGTGGGAGGGAGGAGGGGCCGG	9%
4th G-run, middle tier	CCGGCCAAGGGTGGGAGGGAGGGGGGCCGG	24%
4th G-run, top tier	CCGGCCAAGGGTGGGAGGGAAAGGGGGCCGG	67%
Helical mismatch	CCGGCAAAGGGTGGGAGGGAGGGCGCCGG	82%
Helix-1st G-run, 1st A	CCGGCCTAGGGTGGGAGGGAGGGGGGCCGG	138%
Helix-1st G-run, 2nd A	CCGGCCATGGGTGGGAGGGAGGGGGGCCGG	104%
1st-2nd G-run loop	CCGGCCAAGGGAGGGAGGGAGGGGGGCCGG	114%
2nd-3rd G-run loop	CCGGCCAAGGGTGGGTGGGAGGGGGGCCGG	100%
3rd-4th G-run loop	CCGGCCAAGGGTGGGAGGGTGGGGGCCGG	112%
1-bulge A deleted	CCGGCCAAAGGGTGGGAGGGAGGGGGGCCGG	320%
2-bulge As deleted	CCGGCCAAAGGGTGGGAGGGAGGGGGGCCGG	300%
4th G-run-helix insertion	CCGGCCAAGGGTGGGAGGGAGGGAGGGGCCGG	93%

Mutant oligonucleotides were synthesized and assayed for binding to sulforhodamine agarose. Mutants were analyzed under parallel conditions with the reference oligonucleotide (marked *). Binding efficiency is reported as the fraction of DNA specifically eluted by

sulforhodamine, normalized to that for the reference. Values reported are averaged from five different experiments. Differences between experiments were generally less than 5%. Mutations are in bold; Δ , deletion.

as a result of G-quartet formation [21,22]. To show whether the aptamer functioned as a monomer or as part of a higher-order complex, we examined the efficiency of sulforhodamine agarose binding over a wide range of aptamer concentrations. 5'-End-labeled DNA bound at < 10 nM with approximately the same efficiency as when it was mixed with excess unlabeled DNA to 10 μ M, strongly suggesting that the aptamer folds and functions as a single molecule.

On the basis of the approximately conserved guanine pattern in the loop region, we hypothesized that the aptamers fold to form three-tiered G-quartets. G-quartets have been proposed previously to exist in several DNA aptamers, including those specific for thrombin and for ATP [10,23]. Although high-resolution structural analysis using nuclear magnetic resonance (NMR) of the thrombin aptamer has confirmed the G-quartet model, the ATP aptamer has been shown, instead, to form an extended pseudohelix that creates a binding pocket for two ligand molecules [24,25]. Given this history, we have attempted to test the G-quartet hypothesis biochemically, analyzing both the aptamer's sensitivity to modification by site-specific alkylation agents and its salt requirements for binding.

G-quartet formation is predicated upon a specific pattern of hydrogen bonding that includes pairing between the exocyclic amine of one guanine and the N-7 atom of its

neighbor. Methylation of the N-7 position of guanines by dimethyl sulfide (DMS) makes DNA sensitive to subsequent strand cleavage by piperidine. Accessibility of guanine N-7 positions might therefore be readily probed by this modification reagent. Figure 3 shows the results of chemical probing under conditions in which the folding of the aptamer is altered. Under conditions in which the aptamer is denatured (80°C, 5 mM Hepes), there is no difference in the relative protection of the guanines in the proposed G-quartet and the guanines in the flanking duplex. Under folding conditions (i.e. room temperature and selection buffer), however, the predicted G-quartet guanines are dramatically protected from modification whereas the others are unaffected. No change in modification is apparent upon addition of ligand, suggesting that the structure does not change substantially upon binding or that it re-arranges to form a similarly protected structure. Similar N-7-protection patterns have been observed in other guanine-rich DNAs, which have been shown subsequently, using NMR or crystallography, to form stable quartets [26]. Chemical-modification studies of the ATP DNA aptamer have not been carried out and did not serve as part of the evidence for the initial, incorrect G-quartet model [10].

We sought further evidence for the G-quartet model by examining the aptamer's salt requirements for folding and binding. G-quartets are thought to be specifically stabilized

Figure 3

Demonstrating G-quartet formation in the DNA aptamer. The accessibility of N-7 atoms on guanosines and adenosines was probed by reaction with DMS and DEPC, respectively, as described in the Materials and methods section. (-), no modification reagent added but treated identically otherwise; 80°C, denaturing control, incubated at 80°C with modification reagent; Na, incubated with buffer containing 0.1 M NaCl; K, incubated with buffer containing 0.1 M KCl; K + SR, incubated with buffer containing 0.1 M KCl and 5 mM sulforhodamine B.

by complexation of monovalent cations through up to four guanine carbonyl groups [27]. Studies have consistently shown potassium to be a more effective stabilizer than sodium, especially when the geometry of the G-quartet is constrained by other factors (e.g. short linking loops [21,28]). The original aptamer was selected in buffer that contained 100 mM potassium and ~10 mM

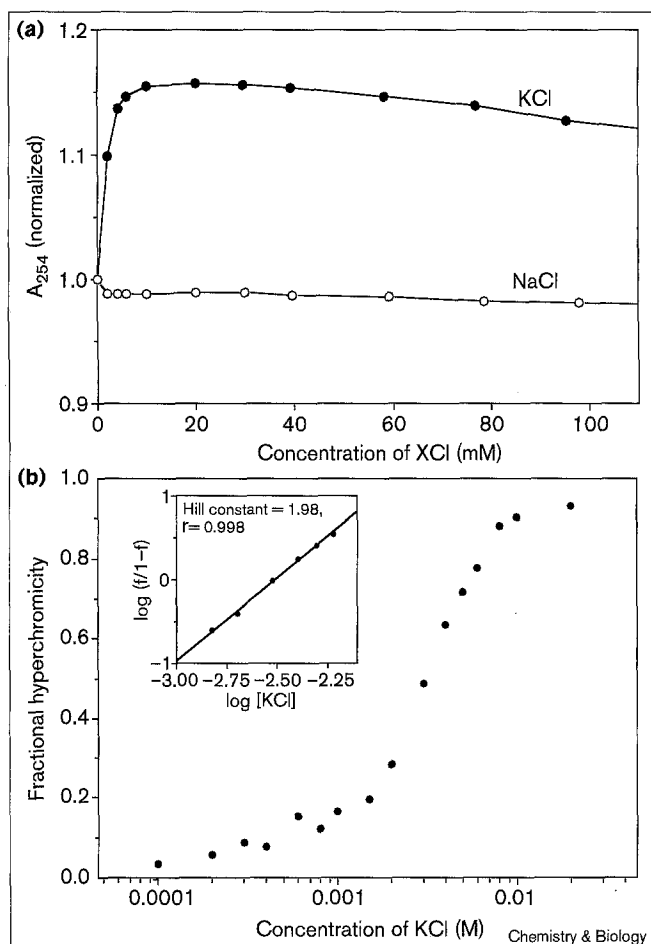
sodium. Virtually no specific binding to sulforhodamine agarose is observed when equilibrated in buffer containing sodium but no potassium (Figure 2c). Probing with DMS also shows that these conditions fail to induce the specific pattern of guanine protections observed in the presence of potassium (Figure 3). To quantify the effect of potassium on DNA folding, we monitored conformational changes using UV absorbance spectroscopy (Figure 4). The addition of 5 mM potassium chloride induced a significant hyperchromic effect (increasing OD₂₆₀ by approximately 15%). No effect was observed upon the addition of sodium, even at a concentration of 100 mM. Detailed measurements of the hyperchromic effect at low potassium concentrations revealed a cooperative transition with a Hill constant of ~2.0 (Figure 4). The simplest interpretation of these results is that two potassium ions bind to stabilize the folding of the sulforhodamine aptamer into its active conformation. Crystallographic studies suggest that monovalent cations are tetrahedrally coordinated between the planes of G-quartets [27]. It is tempting to speculate that single potassium ions are required to bind in each of the inter-quartet spaces to induce folding of the aptamer. Ongoing crystallographic studies aim to test this possibility. It is worth noting that the ATP DNA aptamer that does not form a G-quartet was selected to function in sodium-containing buffer and does not show a potassium requirement for function [10].

In an effort to understand which functional groups on the aptamer are involved in binding the sulforhodamine ligand, we used a combination of both chemical probing and site-directed mutagenesis. As noted previously, guanosines in the G-quartets are highly protected under folding conditions and do not change detectably upon the addition of ligand. Diethylpyrocarbonate (DEPC) modification was used to probe the conformation of adenosines in the loops lying above and below the G-quartets. All adenosines appear relatively accessible to modification, both in the absence and presence of ligand (Figure 3), suggesting that hydrogen bonding to their major-groove faces does not play a role in ligand binding.

To more effectively map the binding site, single substitutions in the sequence of the 29-nucleotide mini-aptamer were prepared and assayed for sulforhodamine agarose binding. To minimize the likelihood that mutagenesis would induce misfolding, we substituted guanosines and adenosines for thymidines, and thymidines for adenosines (avoiding the introduction of cytosines, which might base pair adventitiously with guanosines). The fraction of specifically eluted DNA was determined and used as a measure of the effect of the mutation. Surprisingly, many sites in the aptamer, including all unpaired nucleotides, are tolerant of substitution (Table 1, Figure 5).

Mutations that significantly abolish binding (G15→A, G17→A and G23→A, each reducing binding to <10% of

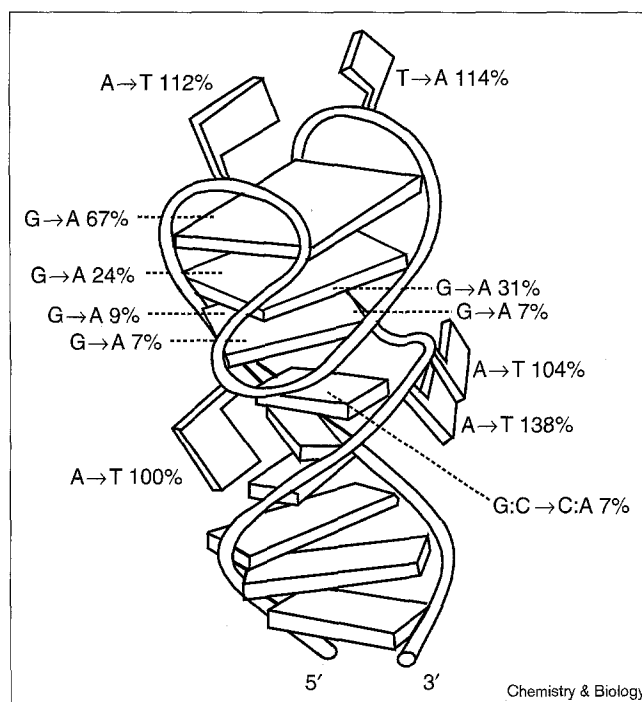
Figure 4



Potassium but not sodium induces a conformational change in the DNA aptamer as monitored by UV absorbance spectroscopy. (a) Aptamer DNA was resuspended in salt-free buffer at $\sim 20 \mu\text{g/ml}$. Increasing amounts of either KCl or NaCl was added and the absorbance was measured after equilibration for 5 min. Absorbance was corrected for dilution by the concentrated salt stock solution and normalized to that for 0 mM salt. (b) KCl titration was carried out as described in (a) but over a much narrower range. The resulting change in absorbance was converted to f , fraction folded, assuming no folding at 0 mM KCl and complete folding at 20 mM KCl. The transformed data was used to generate a Hill plot (inset) to obtain a measure of the cooperativity of potassium-induced folding.

the wild-type DNA) are all clustered in a single proposed G-quartet lying immediately above the Watson-Crick duplex. These nucleotides are probably critical either for proper folding of the aptamer or for making direct contacts with the ligand. To distinguish between these possibilities, we examined the effect of the mutation G17 \rightarrow A on potassium-induced hyperchromicity. In contrast to the strong increase in absorbance observed with the wild-type aptamer, the mutant shows no significant change, arguing that the substitution has simply destabilized the aptamer so that it no longer folds. As such, the effects of mutagenesis on binding activity cannot be used to directly infer the location of the binding site.

Figure 5

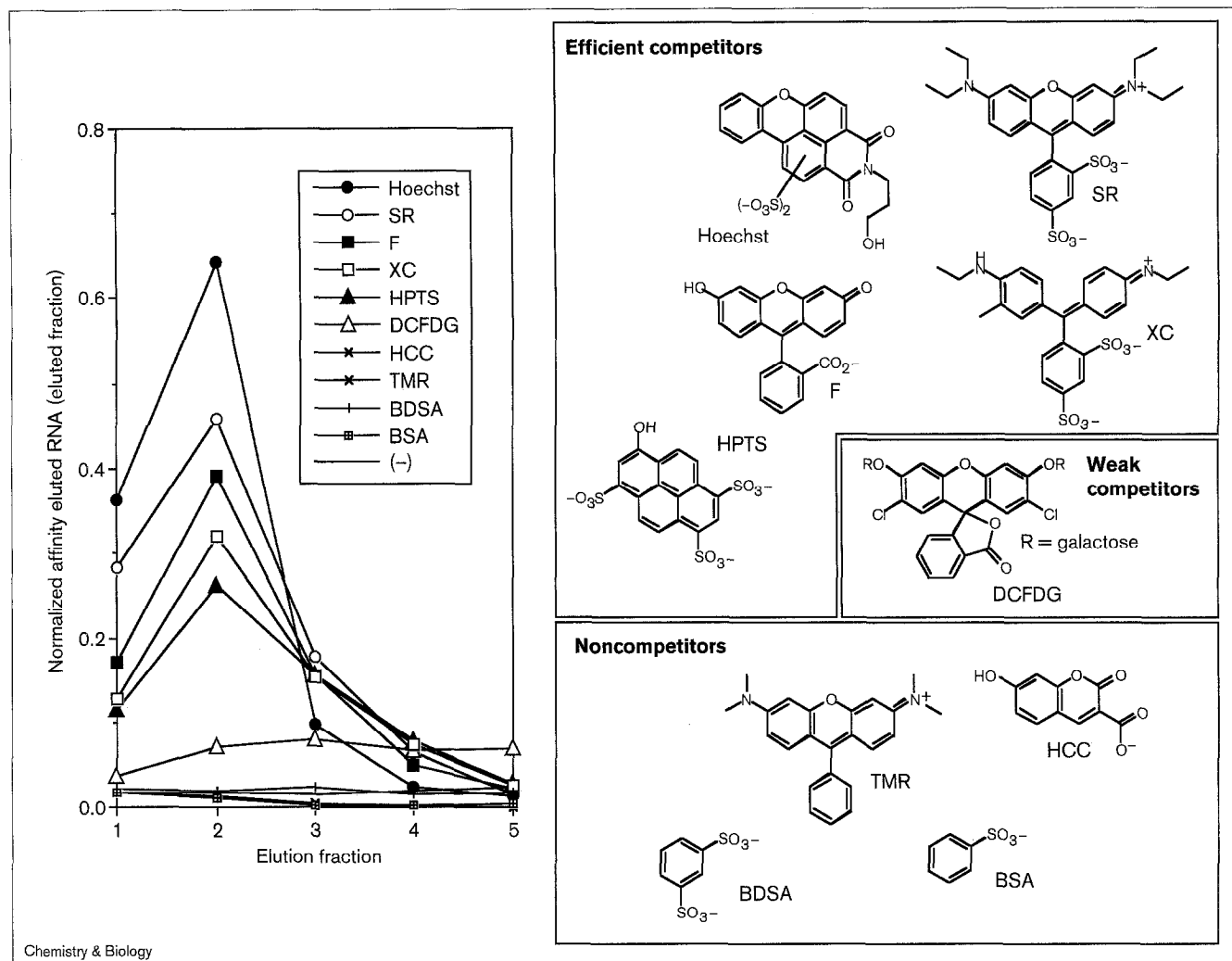


A low-resolution model of the sulforhodamine aptamer based loosely on the thrombin aptamer NMR structure (PDB accession code 1QDH [30]). The effect of site-directed mutations on relative binding efficiency (Table 1) is indicated for each mutated nucleotide.

The fact that loop nucleotide identity has little effect on binding suggests that either the ligand interacts with the loops in a sequence-independent manner or the ligand binds to the G-quartet domain. The finding that the accessibility of adenosine N-7 atoms to DEPC is unaltered by ligand binding (Figure 3) suggests further that these nucleotides are not involved in binding. Deletion of A7 and A8, the nucleotides bridging the duplex and the first run of guanines in the G-quartet domain, actually increases binding substantially. This result would tend to argue against the possibility that a ligand intercalates between the two regions. Deletion of a single predicted G-quartet (converting all of the runs of three guanines to runs of two guanines) abolishes binding but introducing a fourth tier is tolerated. In summary, our experiments have shown that mutations that might disrupt the core structure block binding, mutations that might stabilize it improve binding, and relatively little else has a measurable effect. These results are consistent with a relatively open or ill-constrained binding site that is built largely on the basis of secondary structure rather than specific tertiary interactions.

To understand the basis for the interaction between the ligand and the aptamer, a series of sulforhodamine analogs were tested for the ability to competitively elute minimal sulforhodamine aptamer bound to sulforhodamine agarose.

Figure 6



Specificity of binding was assessed by measuring the ability of sulforhodamine analogs to compete for binding. The graph shows elution fractions obtained after binding the DSR minimal aptamer to sulforhodamine agarose and washing for ten column volumes. Competitors were added to selection buffer at 5 mM concentration (with the exception of DCFDG, added at 1 mM). Hoechst, Hoechst

2495 (benzoxanthene yellow); SR, sulforhodamine; F, fluorescein; XC, xylene cyanole FF; HPTS, hydroxypyrene trisulfonic acid (pyranine); DCFDG, dichlorofluorescein digalactoside; TMR, tetramethylrosamine; HCC, hydrocoumarin carboxylic acid; BDSA, benzene disulfonic acid; BSA, benzene sulfonic acid.

As shown in Figure 6, analogs that contain the complete three-ring xanthene fragment of sulforhodamine are especially efficient competitors of sulforhodamine binding (e.g. fluorescein and Hoechst 2495), whereas those that lack it (e.g. benzene disulfonic acid) appear incapable of binding. Surprisingly, TMR, one of the closest mimics of sulforhodamine, fails to compete for binding. With this exception, all compounds tested that contain three or more aromatic rings function at some level as competitors for binding. This relative lack of specificity is consistent with the results of mutagenesis experiments and could be explained if aromatic stacking, either between tiers of the G-quartet or across its uncapped end, provides the major driving force for binding. Aptamer binding has been quantified both for

immobilized sulforhodamine ($K_D = 190 \pm 20$ nM) and for sulforhodamine free in solution ($K_D = 660 \pm 60$ nM) as described in the Materials and methods section. These values compare favorably to those obtained for most other aptamers with specificity for aromatic ligands (e.g. the ATP RNA aptamer $6-8 \mu\text{M}$ [5] and xanthine $3.3 \mu\text{M}$ [29]) although somewhat tighter binding aptamers have been isolated (e.g., theophylline $0.1 \mu\text{M}$ [7]).

Significance

We have demonstrated that fluorophore-specific DNA aptamers can be isolated using *in vitro* selection and that several aptamers within this pool function as inefficient catalysts to promote multiple rounds of oxidation of a flu-

origenic substrate. A model for the aptamer consisting of a simple three-tiered G-quartet stacked on a duplex agrees with all available biochemical and mutational data. Given its small size and ability to bind a variety of fluorophores and chromophores, several potential uses for such a motif can be envisioned. Its incorporation into other DNAs (e.g. PCR primers) could facilitate their *in vitro* labeling and detection. Mutagenesis followed by both negative and positive selection might yield specialized aptamers optimized for highly specific recognition of individual fluorophores. The current motif could be incorporated into larger random pools that could serve as the starting point for new selection experiments directed at the isolation of ribozymes that utilize fluorogenic substrates. Although the gross structure of the aptamer has been well defined by comparing independent clones in the selected pool, using mutational analysis, chemical modification, and differential absorption UV spectroscopy, the exact location of the ligand-binding site within the aptamer and the forces that drive binding remain open questions.

Materials and methods

Sulforhodamine agarose was prepared by overnight reaction of lissamine B sulfonyl chloride (Molecular Probes, Eugene OR) and adipic acid dihydrazide agarose (Sigma Chemical Co., St. Louis MO), equilibrated in 0.1 M sodium bicarbonate, pH 8.3 and 10% dimethyl formamide. Unreacted fluorophore was removed by extensive washing with DMF and water. The concentration of immobilized sulforhodamine was estimated spectrophotometrically to be ~1 mM.

Single-stranded DNA was prepared by asymmetric PCR amplification. A pool of random sequence DNA molecules with the general sequence AACACTATCCGACTGGCACC-N72-CCTTGGTCATTAGGATCCCG was prepared by conventional solid phase synthesis on a Millipore 8705 DNA synthesizer. DNA was converted to double-stranded form by a large-scale PCR reaction with primers corresponding to the constant sequences listed above. This process effectively enriched the fully extendable, lesion-free sequences present in the original synthesis. Single-stranded DNA was prepared by a tenfold dilution of the PCR reaction into an identical PCR cocktail containing an antisense oligonucleotide to the 3' primer, followed by ten rounds of thermal cycling (linear amplification). DNA was purified by polyacrylamide gel electrophoresis, eluted into water and resuspended in selection buffer (0.1 M KCl, 5 mM MgCl₂, 10 mM Na-HEPES, pH 7.4).

Sulforhodamine-specific DNAs were isolated by passing the equivalent of eight genomes of the DNA pool (240 µg) over an adipic acid dihydrazide pre-column, which subsequently passed onto a 500 µl sulforhodamine-agarose column. The adipic acid dihydrazide column was used in every round in an attempt to minimize enrichment of nonspecific matrix-binding aptamers. After three column washes, the pre-column was discarded and the sulforhodamine-agarose column was washed for an additional 12 column volumes. Specifically bound species were eluted by five column volume washes with selection buffer containing 5 mM sulforhodamine. Eluted fractions were pooled, ethanol-precipitated after the addition of carrier glycogen, and used as template for subsequent PCR amplification as described above for the original random pool preparation.

Individual molecules in the selected pool were cloned by ligating double-stranded DNA from the last round of amplification into a TA-cloning vector (Invitrogen) and transfecting the recombinant plasmids into *Escherichia coli*. Single-stranded DNA corresponding to individual

sequences was obtained by asymmetric PCR amplification of mini-prepped plasmid from isolated colonies.

Single-stranded DNA was assayed for the ability to catalyze the conversion of nonfluorescent DHTMR to TMR. DNA (50 nM – 1 µM) was mixed with 60 µM DHTMR (diluted from a 15 mM stock solution in THF) and 16 mM H₂O₂ in selection buffer and allowed to incubate in the dark at room temperature. In control experiments, reagents were purified by passage over Chelex resin (Sigma Chemical Co., St. Louis) to remove trace transition metals, which might promote DHTMR oxidation. Fluorescence was monitored in a 3 ml quartz cuvette using a Perkin-Elmer L30 fluorimeter at $\lambda_{\text{ex}} = 550 \text{ nm}$, $\lambda_{\text{em}} = 574 \text{ nm}$.

Chemical modification with DMS and DEPC was carried out essentially as described previously [26]. DNA for analysis was 5'-end-labeled with ³²P-γ-ATP and polynucleotide kinase (New England Biolabs). 100 µl of gel-purified, kinased material was treated with either 6 µl DEPC (15 min reaction) or 1 µl of 1:10 diluted DMS (7 min reaction) under various salt conditions at either room temperature or at 80°C (for the denatured control). Reactions were terminated by the addition of carrier tRNA and 0.3 M sodium acetate. DNA was ethanol precipitated, rinsed, and resuspended in 20 µl of 1:10 diluted piperidine containing 1 mM EDTA. After baking for 30 min at 92°C, 20 µl of 2 × gel loading dye was added and the reaction products were resolved on a 20% gel.

The binding constant for immobilized ligand was estimated by measuring the fraction of aptamer bound to sulforhodamine agarose following its dilution to a range of different ligand concentrations. The concentration of sulforhodamine available for binding by the aptamer concentration was estimated by measuring the amount of aptamer specifically bound under saturating conditions (and assuming 1:1 stoichiometry of aptamer : ligand). 20 µl of diluted matrix and trace amounts of kinased aptamer DNA were added to 0.2 micron spin filters (Costar) and diluted with up to 1 ml selection buffer. Following extensive equilibration on a rocker, the dilute matrix slurry was rapidly concentrated by microcentrifugation and immediately washed with 150 µl ice-cold selection buffer. The amount of labeled DNA eluted by equilibration with excess sulforhodamine was determined by scintillation counting. The dissociation constant was determined by plotting bound DNA concentration versus immobilized sulforhodamine concentration and least squares fitting the data to a hyperbolic curve.

The binding constant for ligand free in solution was determined using UV differential absorption spectroscopy. DNA was resuspended in selection buffer to an approximate OD of 0.3 and allowed to equilibrate for 1 h. Dilute sulforhodamine in selection buffer was added in aliquots and allowed to equilibrate for 5 min. Absorption at 255 nm and at 564 nm (representing the absorption maxima for the DNA and the sulforhodamine, respectively) was measured. A standard curve for sulforhodamine was prepared to determine its contribution at 255 nm. After correcting for dilution effects (due to the added sulforhodamine) and for sulforhodamine absorption at 255 nm, DNA absorbance was plotted as a function of sulforhodamine concentration (determined by absorbance at 564 nm). Binding parameters were calculated by least squares fitting to an equation of the form $A = A_0 - \Delta OD \times [SR] / (K_D + [SR])$ where A_0 corresponds to DNA absorbance in the absence of sulforhodamine (SR), ΔOD is the difference in absorbance between the bound and unbound state, and K_D is the dissociation constant.

Acknowledgements

This work was supported by grants from the NIH (GM52707) and the Packard Foundation (C.W.) and by a grant from Hoechst AG to the Department of Molecular Biology, MGH (J.W.S.).

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