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An allosteric synthetic DNA

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ABSTRACT

Allosteric DNA oligonucleotides are potentially useful diagnostic reagents. Here we develop a model system for the study of allosteric interactions in DNAs. A DNA that binds either Cibacron blue or cholic acid was isolated and partially characterized. Isolation was performed using a multi-stage SELEX. First, short oligos that bind either Cibacron blue or cholic acid were enriched from random oligonucleotide pools. Then, members of the two pools were fused to form longer oligos, which were then selected for the ability to bind Cibacron blue columns and elute with cholic acid. One resulting isolate (A22) was studied. Dye- and cholate-binding functions can be separated on sequences from the 5′- and 3′-regions, respectively. Ligand–column affinity assays indicate that each domain binds only its respective ligand. However, the full-length A22 will bind either dye or cholate columns and elute with the other ligand, as if binding by the ligands is mutually exclusive. Furthermore, S1 nuclease protection assays show that Cibacron blue causes a structural change in A22 and that cholic acid inhibits this change. This system will be useful for elucidating mechanisms of allosteric interactions in synthetic DNAs.

INTRODUCTION

Allosteric regulation is a common phenomenon responsible for the reversible control of many proteins (1,2). In this mechanism, small regulatory molecules bind at sites other than the enzymatic active sites and cause protein conformational changes that alter enzymatic activities. This form of regulation allows for the sensitive modulation of biochemical activities in response to critical metabolites. Because it is found in the majority of biochemical pathways and is essentially ubiquitous among organisms, allosteric regulation is proved to be an extremely useful regulatory strategy. Among natural systems, allosteric regulation has been conclusively shown only within the protein macromolecular class. However, we are only beginning to understand the richness of the structural and functional roles of nucleic acids. It is clear that the catalytic and binding activities of nucleic acids depends on structure, which can be readily altered by changes in the solvent, temperature and ionic conditions (3). This apparent plasticity strongly suggests that allosteric mechanisms will be found to regulate at least some nucleic acid functions. In addition, allosteric nucleic acids have the potential for development as diagnostic reagents. Experimental models will be valuable for determining the structural and mechanistic principles for allosteric nucleic acids. Such information will be of both basic and practical interest.

Recently, two kinds of allosterically controlled hammerhead ribozymes have been constructed by the fusion of ligand-binding domains to helix II of the ribozyme. In the first case, Tang and Breaker generated several fusions to an ATP-binding aptamer (4–6). In certain of the fusions, ATP inhibits ribozyme activity by up to 100-fold. Structural modeling shows that in these constructs, but not in the uninhibited fusions, the ATP-stabilized ATP domain sterically blocks proper folding of the ribozyme (6). In the second case, Araki *et al*. (7) made several modified ribozymes by fusing an FMN-binding aptamer while leaving different sized linkers between the FMN and ribozyme domains. Fusions with short helix II linkers are generally poor ribozymes, but their cleavage activities can be substantially increased by FMN, presumably because it stabilizes the FMN–helix II region in a conformation compatible with catalysis. In both of these examples, ribozyme activity is modulated by ligand-induced changes in macromolecular structure, which is the hallmark of allosteric regulation. Furthermore, these two kinds of allosteric ribozyme are regulated by completely different structural mechanisms. It is highly likely that further mechanisms will be found to be operable for allosteric nucleic acids.

Here, we describe a novel experimental system for the isolation and characterization of allosteric synthetic DNA molecules. Although RNA is commonly used for nucleic acid structural and functional analyses, many DNA aptamers and DNAzymes have also been isolated and characterized (8–14). Many of the technological approaches and methods are applicable to the study of both classes of nucleic acids and, more importantly, the structural and functional properties of RNA and DNA are also largely comparable. We prefer DNA as a model system because its relative stability makes DNA more suitable for *in vitro* diagnostic reagents (9). Furthermore, SELEX (15,16) with DNA is technically simpler because it does not require reverse transcription steps. This makes it relatively easy to isolate allosteric aptamers via complex SELEX schemes in which molecules that have different functions are selected separately and then fused to create composite molecules that have interacting functions. We used such a scheme to isolate a DNA molecule that binds either a visible dye (Cibacron blue) or a simple steroid (cholic acid). Each ligand appears to inhibit the binding of the other. Furthermore, we show that cholic acid inhibits Cibacron blue binding through an allosteric mechanism, although the precise structural details of the mechanism are not yet clear. These data show that allosteric DNAs are indeed possible. This work also provides materials for the study of the capacity of DNA to form specific binding domains for non-nucleotide substrates and for determining principles that can contribute to allosteric mechanisms in nucleic acids.

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MATERIALS AND METHODS

DNA pools for SELEX

Random single-stranded (ss)DNA pools and primers were synthesized at the Wake Forest University School of Medicine DNA synthesis core facility. The synthesized pools of DNA had the sequences: pool I, 5′-GTCGAATTCGCACGCTTGTCAG-$(N)_{28}$-AGCTTAGGATCCAACCTGATCT-3′; pool II, 5′-AGCTT-AGGATCCAACCTGATCT-$(N)_{36}$-GGTACCAACTGCATACC-GAGCT-3′ (N stands for an equimolar mixture of A, C, G and T).

Sequences of the primers were: P1, 5′-GTCGAATTCGCACG-CTTGTCAG-3′; P2, 5′-AGATCAGGTTGGATCCTAAGCT-3′; P3, 5′-AGCTTAGGATCCAACCTGATCT-3′; P4, 5′-AGCTC-GGTATGCAGTTGGTACC-3′. Primers P1 and P2 can amplify pool I, while P3 and P4 can amplify pool II. Also, note that the 3′-end of pool I is identical to the 5′-end of pool II. Therefore, the two pools can be fused by annealing pool I to the complement of pool II, followed by primer extension to make the fused sequences double-stranded. P1 and P4 can be used to amplify the fused pool and P1 can be used for asymmetric PCR.

PCR and asymmetric PCR

Reactions contained up to 1 pmol template and from 50 to 100 pmol primer(s). Standard PCR to obtain double-stranded DNA products used primers for each strand of the target sequence. Asymmetric PCR to obtain single-stranded products were performed using only a single primer. Samples were prepared in 50 µl volumes containing 10 mM Tris–HCl, pH 8.6, 50 mM KCl, 2 mM $MgCl_2$, 200 µM each dNTP and 0.05 U/µl *Taq* DNA polymerase (Promega). Samples were treated with from 5 to 20 cycles of 96°C for 30 s, 60°C for 1 min and 72°C for 1 min. Amplified products were banded on 12% native polyacrylamide gels and DNAs were eluted from gel slices by crushing and soaking (for at least 6 h) in a buffer containing 10 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.5 M NaCl. After filtration to remove the insoluble gel matter, DNAs were precipitated with 2.5 vol of ethanol, recovered by centrifugation and resuspended in 100 µl of 1 mM Tris–HCl, pH 8.0, 0.1 mM EDTA.

Selections

Ligand-binding DNA aptamers were selected by passing the DNA pool over Cibacron blue (pool I) and cholic acid (pool II) affinity columns (Sigma). For each column, 0.5 ml of column resin was pre-equilibrated with ~2 ml of column buffer (500 mM LiCl, 1 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.0). Approximately 100 pmol of ssDNA in 60 µl of column buffer was heated to 95°C for 5 min, allowed to cool to room temperature for 20 min and then loaded onto the column. After incubation for 30 min to allow for binding, the column was washed with 5 ml of column buffer. Bound species were eluted with 2 ml of 2 mM EDTA (Cibacron blue columns) or 5 mM cholic acid in column buffer (cholate columns). Eluted material was collected, concentrated by butanol extraction, then glycogen (as carrier) was added to 20 µg/ml and the DNA was precipitated with 3 vol of ethanol. Precipitates were dissolved in 20 µl of H_2O and DNAs were divided into four PCR reactions. Selections were monitored by scintillation counting and progress was assessed using the strategies described by Yarus (17).

5′-End-labeling of DNA

About 10 pmol of DNA were 5′-end-labeled using 100 µCi [γ-^{32}P]ATP (166 mCi/ml, specific activity, 7000 Ci/mmol) using 10 U T4 polynucleotide kinase (New England Biolabs). Reaction conditions were 70 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT in a 20 µl volume and mixtures were incubated for 1 h at 37°C. The labeled products were isolated from residual [γ-^{32}P]ATP by passage through Sephadex G-50 spin columns equilibrated with 10 mM Tris, pH 7.5, 0.5 M KCl.

Cloning

PCR-amplified DNA from the final round of selection was cloned using standard methods (18). The *Eco*RI and *Kpn*I cloning sites are encoded in the primer regions. The plasmid vector was pLW1, which contains the P1 and P4 primer sequences cloned into a derivative of pET3a (19). DNA sequencing was performed as described (20).

Structural probing with S1 nuclease

S1 nuclease digestions were performed as described (21), with modifications. Approximately 10 pmol of the 5′-end-labeled ssDNA was heated to 90°C for 5 min in 8 µl of column buffer (0.5 M LiCl, 20 mM Tris–HCl, pH 7.6, 1 mM $MgCl_2$) and cooled slowly at room temperature for 20 min. Then, cholic acid and/or Cibacron blue were added to the appropriate samples and all samples were incubated at room temperature for at least 2 h. Then, 2 µl of S1 nuclease mixture (500 mM sodium acetate, pH 4.5, 100 mM $MgCl_2$, 500 mM KCl, 10 mM $ZnSO_4$ and 100 U S1 nuclease) was added to each sample and the samples were incubated for 30 min at room temperature. Reactions were terminated by the addition of 10 µl of gel loading buffer (95% formamide, 5 mM EDTA, 1 mg/ml each of bromophenol blue and xylene cyanol) and heating at 95% for at least 5 min. For analysis, samples were fractionated on 10% denaturing polyacrylamide gels.

RESULTS AND DISCUSSION

Selection of ssDNAs that have apparent allosteric properties

Our goal was to isolate DNAs that specifically bind both Cibacron blue and cholic acid, but not at the same time. We chose these ligands because they both have many possible hydrogen bond donor and acceptor groups as well as planar surfaces for stacking interactions. Cibacron blue-binding DNAs have been previously isolated (9). It seemed to us that the most useful system would have the two binding activities in interacting but separable sequences. This would simplify analyses by allowing study of separated sequences with the individual functions, and it would also facilitate future ‘domain swap’ experiments. To isolate such molecules, we designed the selection in five stages, which are illustrated in Figure 1.

In the first stage, we used SELEX to enrich for ssDNA pools that are capable of binding one or the other ligand. Selection was performed by affinity chromatography to columns that contain either Cibacron blue (for pool I oligos) or cholic acid (pool II). Retained ssDNA was eluted from the cholate column with column buffer containing 5 mM cholic acid. We were unable to use Cibacron blue in the elution buffer to select ssDNA bound to dye columns because residual dye in the eluate inhibited PCR. Instead, ssDNA was eluted from the dye column with 2 mM EDTA. DNAs in the eluates were amplified by PCR and then

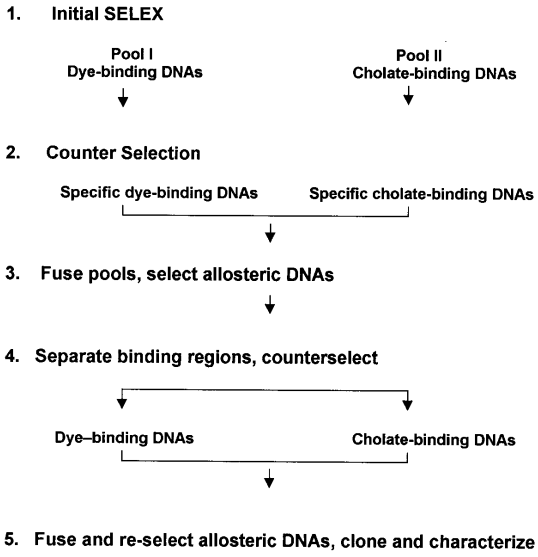

Figure 1. Flow chart of the selection scheme. See text for details.

ssDNAs were prepared from the products by asymmetric PCR. After five and six rounds of selection, respectively, large fractions of the enriched pool I and pool II oligos were retained on the dye and cholate columns.

In the second stage, the two enriched pools were treated to remove molecules that have high affinities for the column matrix or that cross-react with the other ligand. For the dye-binding pool, ssDNA from the fifth round was passed over a cholate column. Sequences retained on this column (~2% of input) were discarded and the flow-through fraction was re-applied to a Cibacron blue column. The column was washed with 6 column vol of column buffer containing 5 mM cholic acid to wash out those molecules that are eluted with cholate. Then the retained DNA (~28%) was eluted with 2 mM EDTA, precipitated with ethanol, amplified by PCR and used as the purified dye-binding pool. Similarly, specific cholate-binding molecules were purified by passing the sixth round pool over a dye column to remove molecules that bind to Cibacron blue or the affinity column matrix. About 3% of the pool was retained on the dye column and discarded. The flow-through fractions were re-applied to a cholic acid column. The column was washed with 6 vol of 5 mM Cibacron blue to wash out molecules that could be eluted with the dye. The retained DNA (~62%) was eluted with 5 mM cholic acid, precipitated, amplified and then used as the purified cholate-binding pool. The two pools were expected to be largely free of oligos that bind strongly to either the column matrix or to the other ligand.

In the third stage, the dye-binding and cholate-binding domain pools were linked by a PCR protocol and the resulting pool was selected for allosteric interactions. The 3′-end of pool I is identical to the 5′-end of pool II so that the two pools can be fused by PCR (Materials and Methods). Single-stranded copies of the fused pool were subjected to six rounds of SELEX, enriching for molecules that bind the dye column and elute with 5 mM cholic acid. Although the separated dye-binding pool has a high affinity for the dye column, the fused pool initially had a low affinity. These results are consistent with those of a similar experiment by Burke and Willis (22). They found that the fusion of RNA aptamers selected for different functions was usually accompanied by large decreases in ligand binding. This they attribute to the formation of alternative structures that compete with the effective ligand binding structures. By the sixth round of selection, however, 38% of the input ssDNA was retained through extensive washing and then eluted with a pulse of cholic acid.

In the fourth stage, the individual dye-binding and cholate-binding sequences were isolated and again subjected to counter-SELEX to remove undesirable binding activities. The dye-binding and cholate-binding sequences were separately amplified by PCR. To remove oligos that have high affinities for either the column matrix or the other ligand, ssDNAs were twice passed over columns containing the other ligand. The flow-through fractions were collected and amplified. The resulting pools are expected to be very specific for their respective ligands and also to have properties that facilitate allosteric interactions when fused to at least some members of the other domain pool. It is not clear that this counter-SELEX stage was necessary, but it seemed prudent to perform these simple protocols because they are likely to decrease the number of clones that need to be characterized to find those that contain the desired allosteric properties.

In the fifth and final stage, the two domains were again linked and carried through SELEX as in stage three. After five rounds of SELEX, the pool was cloned and individual isolates were characterized.

Relative allosteric activities of individual isolates

From a total of 45 clones, we obtained 19 novel sequences. There was no consensus sequence for the dye-binding region; this is consistent with the results of Ellington and Szostak (9), who also found that a wide variety of sequences were apparently capable of binding Cibacron blue. For the cholate region, there was one consensus sequence represented by four members. In addition, two isolates had the same cholic acid region but different dye domain sequences. Because of their relatively high abundance in the pool, these six sequences were chosen for a comparative analysis of their allosteric activities. The synthetic sequence regions were lifted from the plasmids as PCR products and then single-stranded sequences were prepared by asymmetric PCR using the double-stranded products as templates (Materials and Methods).

Oligos were bound to Cibacron blue columns and relative allosteric activity was estimated by measuring oligo release by an eluant containing 5 mM cholic acid. The results (Table 1) show that oligos vary greatly in both their dye-binding and apparent allosteric properties. Isolate A22 has a relatively high affinity for the column and also has the strongest apparent allosteric property. About 40% of the input ssDNA bound to the column, indicating that at least that fraction was competent to bind the column. Most of the bound A22 (37% of input) was released by cholic acid. This isolate was chosen for more detailed binding and structural analyses to confirm an allosteric property.

The primary structure of A22 is shown in Figure 2. It contains 127 nt, which is 3 nt shorter than expected from the input oligos. Apparently, 1 and 2 nt, respectively, were deleted from the randomized dye and cholate regions during the selection protocols.

Analyses of the binding and apparent allosteric properties of A22

In order to determine whether the apparent allosteric property of A22 requires both binding motifs, the allosteric properties of the full-length A22 were compared with those of the isolated dye-binding and cholate-binding sequences. Sequences containing

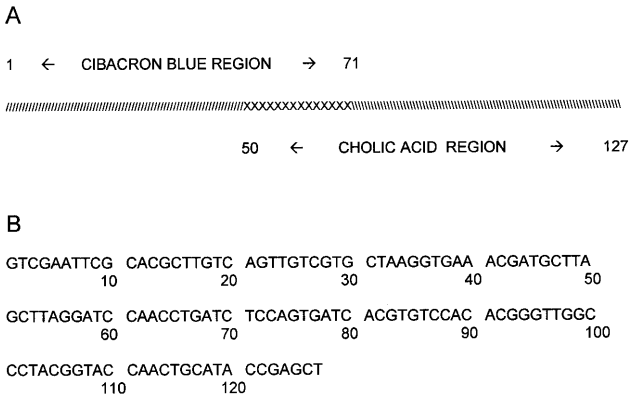

Figure 2. Primary structure of A22. (**A**) A22 contains 127 nt. The regions selected for Cibacron blue binding and cholate binding are at the 5′- and 3′-ends, respectively, and share a 22 nt overlap region including positions 50–71. (**B**) The nucleotide sequence of A22.

the individual binding activities were recovered from A22 by PCR and single-stranded copies were made by asymmetric PCR (Materials and Methods). Then, ssDNAs were loaded onto affinity columns, which were then washed with 10 vol of loading buffer and, finally, the columns were washed with column buffer containing the other ligand (at 5 mM) to elute allosteric DNA.

Table 1. Binding and allosteric properties of individual isolates

Isolate	Bound to dye column	Eluted with cholic acid
A2	34	8
A8	32	18
A9	30	10
A14	28	14
A10	35	20
A22	40	37

Approximately 60 pmol of ^{32}P-labeled ssDNA was loaded and eluted as described in the text. ssDNAs were prepared by asymmetric PCR using restriction digested plasmids as templates. All values are percent input DNA.

The separated dye-binding domain binds to the dye column slightly better than does A22 (Fig. 3A), as if fusion to the cholate-binding domain reduces dye binding by the dye-binding domain. Cholic acid does not significantly elute the separated dye-binding domain, but it effectively elutes the full-length A22. Together, these data confirm the apparent allosteric behavior of A22.

Similar results were observed for the comparison of A22 with the isolated cholate-binding sequence (Fig. 3B). The isolated cholate-binding motif bound to the cholic acid column with a slightly higher affinity than A22, as if the addition of the second sequence reduces binding by the cholate-binding sequence. Although not selected for it, A22 is released from the cholic acid column by Cibacron blue, which is consistent with an allosteric mechanism in which dye and cholate binding are mutually exclusive. Binding by the separated cholate-binding domain was not affected by 5 mM Cibacron blue.

Together, the data in this section suggest that although the two overlapping sequences are specific for their ligands, the joint A22 may form mutually exclusive structures that favor the binding of

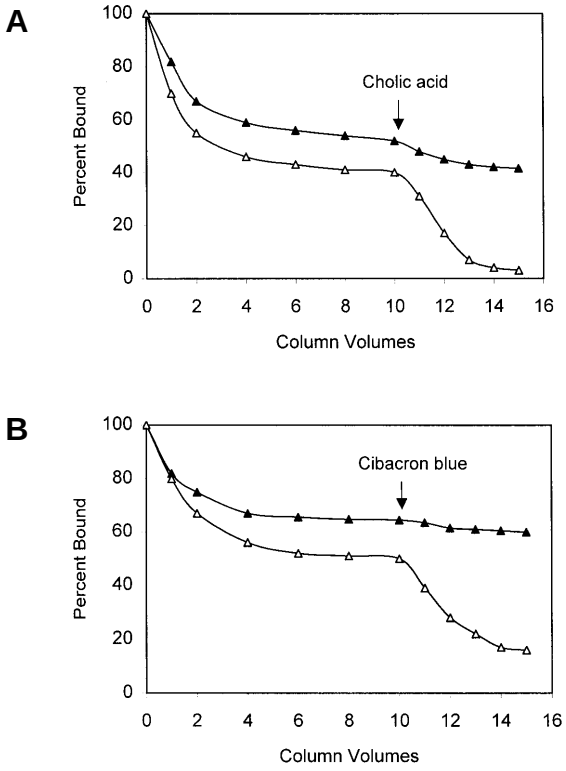

Figure 3. Column binding and elution profiles of A22 and isolated dye- and cholate-binding sequences. DNAs were added to columns, which were then washed with 10 column vol to remove unbound DNA. Then, bound DNAs were eluted with column buffer containing the other ligand (e.g. cholic acid to elute from the dye column; fractions that contain the other ligand are to the right of the arrows). The data points for the isolated ligand-binding sequences and A22 are represented by filled and open symbols, respectively. (**A**) The Cibacron blue column; (**B**) cholic acid column.

either the dye or cholic acid. It is likely that either ligand stabilizes the corresponding bound structure at the expense of the other structure or structures.

Evidence of allosteric changes within A22

If cholate inhibits binding by Cibacron blue, then cholate should inhibit dye-induced changes in A22 structure. To detect any such effects, S1 nuclease was used to probe A22 structure. Nuclease S1 is a single strand-specific endonuclease and the products of a partial S1 nuclease digestion allow the identification of regions that are predominantly single-stranded (21). Probing was carried out during incubation in the presence of each ligand alone and in combinations of the ligands at various ratios (Materials and Methods). A representative gel is shown in Figure 4. For technical reasons, we were unable to probe ~15 nt at the 5′-end of the oligo.

Cibacron blue changes the S1 nuclease susceptibility pattern of A22, with the most dramatic effect being substantial increases in the susceptibility of nt 62–67 (lanes 3–7). It seems likely that dye binding alters A22 structure such that these nucleotides become predominantly single-stranded; the data do not, however, allow us to identify the specific secondary or tertiary structural changes. This effect is readily apparent at only 10 µM Cibacron blue (lane 7) and, because all of the samples with higher dye concentration have virtually identical patterns (lanes 3–6), it is

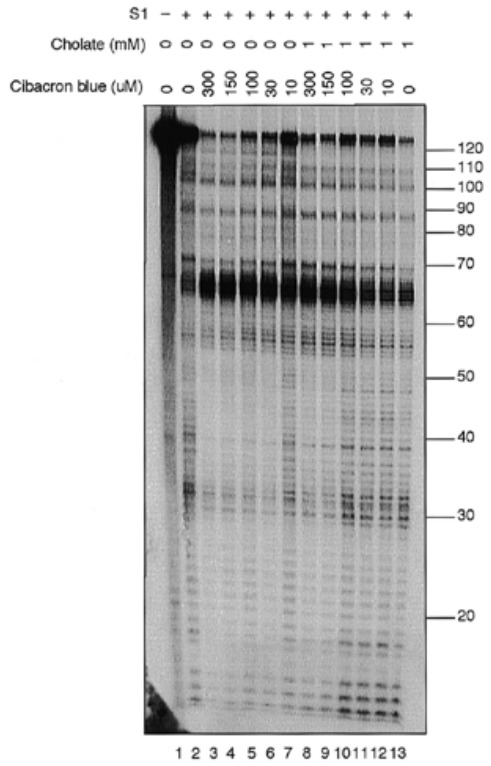

Figure 4. S1 nuclease analyses of A22. Samples were prepared as described in Materials and Methods. Sample treatments are indicated above each lane. Nucleotides are numbered from the 5′-end and are indicated at the right.

likely that A22 is saturated with dye at or above 30 µM. However, in the presence of 1 mM cholic acid, this effect requires at least 100 µM dye (lanes 8–12). Thus, cholate inhibits this dye-induced effect on A22 structure. The most likely explanation is that Cibacron blue and cholate compete for alternative A22 conformations and, therefore, that higher dye concentrations are required to saturate the oligo in the presence of 1 mM cholic acid.

Together, the column elution data and the S1 probing analyses provide strong evidence that cholate and Cibacron blue compete for alternative structures of A22, which therefore has allosteric activities. This initial analysis does not allow us to determine the specific structural basis for allosteric effects. However, it is noteworthy that the nucleotides that are most strongly affected by dye binding (nt 62–67) are in the overlap region used to fuse the dye- and cholate-binding sequences (Fig. 2) and are, therefore, present in each of the separated binding sequences. We do not know whether this region is required for cholate and dye binding, but if alternative conformations of a shared sequence are required for these activities then negative allosteric interactions would be virtually guaranteed. Studies to conclusively determine the allosteric mechanism are underway.

CONCLUSION

We have isolated and characterized allosteric DNAs that tightly bind a Cibacron blue affinity column and then elute with a pulse of cholic acid. Individual isolates with this apparent allosteric activity differ in their affinities for the dye column and in their allosteric properties. Binding and preliminary structural analyses of one oligo indicate that Cibacron blue and cholic acid probably compete for alternative structures, but the precise molecular mechanism of the allosteric interactions cannot yet be resolved.

Allosteric DNAs may be of tremendous practical use. Like RNA and protein reagents, DNA aptamers are clearly capable of specific binding and catalysis (8–14), but DNAs have the distinct advantage of being intrinsically much more stable than RNA and most proteins. Thus, DNAs may prove valuable as diagnostic reagents, especially in cases where an extended shelf-life is necessary or storage conditions are problematic. An understanding of the biochemical mechanisms of binding and allosteric regulation will facilitate the development of these reagents.

This novel experimental system will be useful for dissecting the molecular details of allosteric interactions in synthetic DNA reagents. The preliminary characterization presented here shows that the fundamental mechanisms of DNA aptamer function may be accessible through these reagents. Furthermore, an experimental system that uses steroids as ligands may facilitate development of oligo-based assays for important biomarkers. The steroids include many similar compounds that have different functions and whose individual concentrations are physiologically important. RNA aptamers that are able to discriminate between similar compounds (23,24) give optimism that it may be possible to isolate allosteric aptamers specific for individual steroids. Not only would these reagents be of specific biotechnological use, but this system could provide general information on how readily DNA-based reagents can be tuned for discrimination among similar compounds.

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