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On the Magnitude of the Chelate Effect for the Recognition of Proteins by Pharmacophores Scaffolded by Self-Assembling Oligonucleotides

Samu Melkko,[1,2] Christoph E. Dumelin,[1,2] Jörg Scheuermann,[1] and Dario Neri[1,*]
[1] ETH Zürich
Institute of Pharmaceutical Sciences
Wolfgang-Pauli-Str. 10
8093 Zürich
Switzerland

Summary

The simultaneous interaction of the binding moieties of a bidentate ligand on adjacent epitopes of a target protein represents an attractive avenue for the discovery of specific, high-affinity binders. We used short DNA fragments in heteroduplex format to scaffold pairs of binding molecules with defined spatial arrangements. Iminobiotin derivates were coupled either via bifunctional linkers or by using various oligonucleotides, thus allowing monovalent or bivalent binding to *streptavidin*. We determined the binding affinities of the synthesized constructs in solution. We also investigated the efficiency of recovery of superior bidentate ligands in affinity capture experiments, by using both radioactive counts and DNA microarrays as readouts. This analysis confirmed the suitability of the DNA heteroduplex as a scaffold for the identification of synergistic pairs of binding moieties, capable of a high-affinity interaction with protein targets by virtue of the chelate effect.

Introduction

The isolation of molecules capable of high-affinity and specific binding to a biological target is a central problem in chemistry, biology, and pharmaceutical sciences. Recent approaches to the solution of this problem include the generation of large combinatorial libraries [1], the fragment-based discovery of bidentate ligands to protein targets [2], as well as the generation of large DNA-encoded chemical libraries [3–8]. Heteroduplex DNA structure may facilitate the self-assembly of pharmacophores and provide unique oligonucleotide sequences as "barcodes" for the identification of preferential binding molecules [3, 8, 9]. In most cases, high-affinity binders rely on the simultaneous engagement of adjacent binding sites of a target protein. However, the contribution of this chelate binding mode to the overall affinity is largely unexplored. We used *streptavidin* and iminobiotin as a model system by which to investigate to what extent chelate binding on a target protein by bidentate ligands contributes to a high-affinity binding interaction.

Results and Discussion

In contrast to biotin, iminobiotin binds to *avidin* (and less efficiently to *streptavidin*) with a moderate affinity constant, in a pH-dependent manner [10]. *Streptavidin* is a homotetrameric protein that contains two pairs of adjacent binding pockets for iminobiotin (≈20 Å apart) on opposite sides of the homotetramer [11]. We chemically conjugated either iminobiotin or a cyanine dye (used as negative control) at the 5′ or 3′ extremity of amino-modified oligonucleotides, and we used the resulting conjugates in heteroduplex format in binding experiments with *streptavidin* (Figure 1A). At the 5′ end of the oligonucleotides, we used three linkers of different length that carry a terminal primary amine (Figure 1B). At the 3′ end, we used three structurally different linkers (Figure 1B), which resulted in different exit vectors for pharmacophores attached to amino-tagged oligonucleotide heteroduplexes (Figures 1C and 1D). In particular, the use of linker z, in which the iminobiotin emerges from an additional 3′ terminal base moiety, allowed for the preparation of DNA fragments in which the exit vectors for the attached chemical moieties are essentially parallel.

We used band-shift experiments [12, 13] to determine the dissociation constant (K_d) in solution for complexes between oligonucleotide derivatives in a stable heteroduplex format (24 bases) and *streptavidin* as target protein. In these experiments, a complex is allowed to form in solution; once equilibrium is reached, the unbound DNA fragment and the DNA-protein complex are separated by native gel electrophoresis. The relative ratios of bound and unbound DNA fragments allow for the determination of an apparent K_d value.

The spatial arrangements of iminobiotin moieties, resulting from the combination of different iminobiotin-DNA linker structures, contributed to different binding affinities to *streptavidin*. While no complex formation between monodentate iminobiotin-DNA derivatives was observed at concentrations as high as 100 μM, stable complexes with *streptavidin* could be observed for all bidentate iminobiotin derivatives (Figure 2). The highest binding affinity originated from the combination of linker z at the 3′ end, and linkers b or c at the 5′ end, with an apparent dissociation constant to *streptavidin* of 140 nM, indicating an apparent affinity gain mediated by the chelate effect of at least a factor of 700. Hamblett and colleagues [14] had previously reported a 640-fold gain in kinetic stability of bidentate biotin derivatives to the Y43A *streptavidin* mutant, which displays a modest binding affinity to biotin and monovalent biotin derivatives.

The self-assembly of DNA oligonucleotides carrying individual chemical compounds at their extremities enables the facile generation of large chemical libraries from smaller sublibraries [8, 15]. The isolation of binding molecules to target proteins of choice from such self-assembled heteroduplex libraries or from other DNA-encoded libraries [3–7] entirely relies on the specific enrichment of binding compounds from the library compounds, with a clear correlation between enrichment and binding affinity.

The band-shift experiments shown in Figure 2 clearly indicate that the linker structures used to connect binding moieties and the DNA heteroduplex greatly influence the resulting binding affinity of bidentate ligands for the

*Correspondence: neri@pharma.ethz.ch
[2] These authors contributed equally to this work.

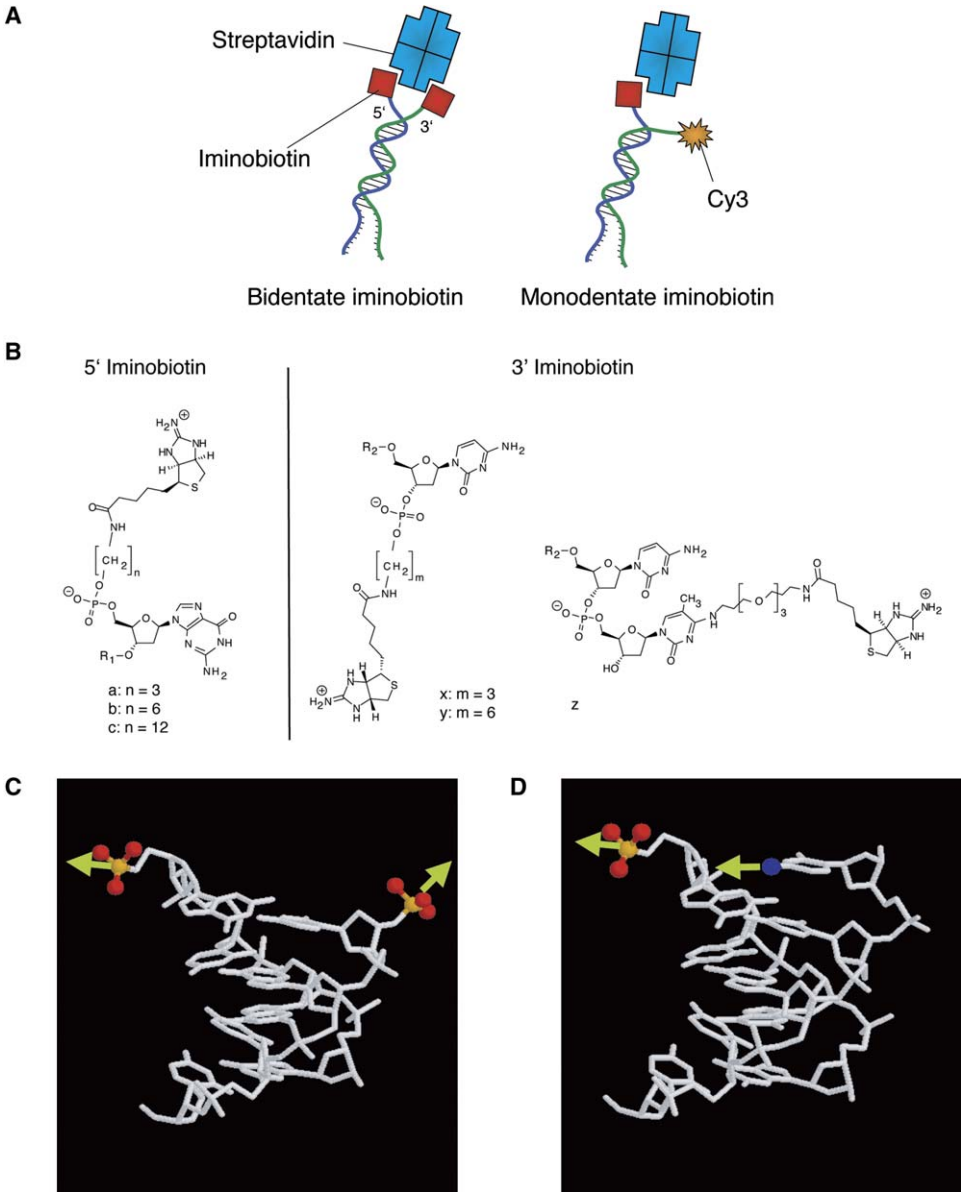

Figure 1. Bidentate Ligands Scaffolded by Heteroduplex DNA

(A) Schematic representation of a bidentate iminobiotin ligand simultaneously interacting with adjacent iminobiotin binding sites of a *streptavidin* tetramer and of monodentate binding. Dimerization is mediated by double-strand formation of DNA oligonucleotides that are covalently coupled to iminobiotin or a cyanine dye (used as negative control) on their 5′ or 3′ termini, respectively.

(B) Structure of different primary amino modification linkers of DNA oligonucleotides. a, b, c: 5′-amino modifications with different linkers originating from the 5′ phosphate group of the 5′ terminal deoxyguanosine nucleotide, R_1: 5′-gagcttctgaattctgtgtgctg-3′. x, y: 3′-amino modifications with linkers originating from the 3′ phosphate group of the 3′ terminal deoxycytidine, R_2: 5′-cagcacacagaattcagaagctc-3′. z: 3′ amino modification originating from additional base at the 3′ terminus.

(C and D) Three-dimensional models of the extremities of a DNA segment (B-form) from which linkers to organic molecules originate ("exit vectors"). (C) 5′-amino-modified oligonucleotide (formats a, b, c) paired with a 3′-amino-modified oligonucleotide (formats x, y). (D) 5′-amino-modified oligonucleotide (formats a, b, c) paired with a 3′-amino-modified oligonucleotide (format z).

corresponding protein target (Figure 1). Since affinity selection methodologies crucially rely on the preferential recovery of higher-affinity binders, we studied how the improved affinity of bidentate ligands influences their recovery. For this reason, we performed panning experiments with *streptavidin* and (mono- or bidentate) iminobiotin derivatives, and we quantified the enrichment factors in the selections either by radioactive counting or by using DNA microarrays.

^{33}P-labeled oligonucleotide derivatives carrying iminobiotin and/or cyanine dyes as chemical moieties at their extremities were allowed to form a double-stranded DNA structure and were incubated with *streptavidin*-sepharose beads. After repeated rounds of washing, aliquots of the input, of the flowthrough, of the wash fractions, and of the oligonucleotides recovered after selections were subjected to radioactivity counting. For comparison, an excess of free iminobiotin

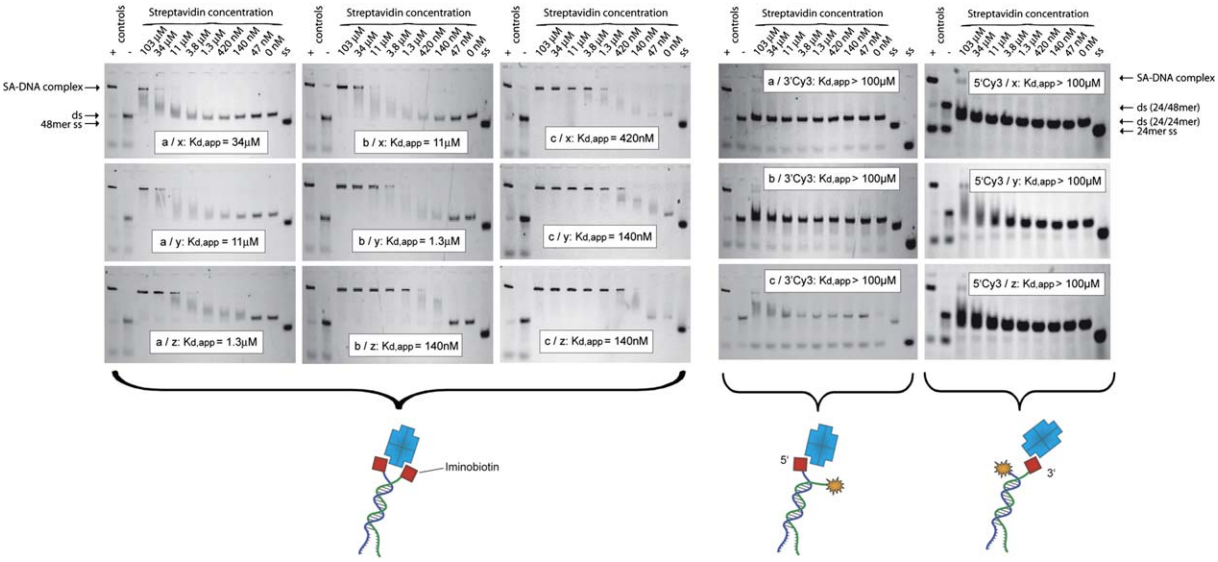

Figure 2. Affinity Determination by Band-Shift Experiments

Affinity determination by band-shift experiments of oligonucleotides conjugated with iminobiotin to tetrameric *streptavidin*. Gel images were recorded by fluorescence imaging utilizing Cy3-labeled oligonucleotides. Bidentate iminobiotin ligands (as in Figure 1A) in all possible combinations between 5′ iminobiotin (linker structures a, b, and c) and 3′ iminobiotin (linker structures x, y, and z) were assayed. Monodentate 5′ iminobiotin with linker structures a, b, and c, as well as monodentate iminobiotin with linker structures x, y, and z, was assayed (paired with a Cy3-labeled oligonucleotide). All bidentate iminobiotin derivates show a higher affinity to *streptavidin* in the band-shift assay compared with the monodentate derivates.

was added to the samples after incubation of the double-strand oligonucleotides with *streptavidin* sepharose in order to increase the stringency of selection, favoring ligands with higher kinetic stability.

Figure 3 shows that bidentate iminobiotin derivatives are preferentially enriched (63.2% recovery) in affinity selections, compared to monodentate iminobiotin ligands (17.1% and 21.1% recovery) and to irrelevant chemical moieties (0.21% recovery). Increasing the stringency of the selection by adding 1 mM free iminobiotin did not substantially lower the recovery of the bidentate ligand (16.9% recovery), while the recovery of monodentate ligands (1.1% and 1.8%) was reduced almost to background levels (0.22%).

In affinity selections, based on encoded self-assembling chemical libraries [8], the identification of higher-affinity binders from a library of compounds typically relies on the use of oligonucleotide microarrays, which display stronger fluorescence signals for those compounds that are preferentially enriched. In order to study whether the capture of iminobiotin ligands from a library of compounds can be detected on oligonucleotide microarrays, we paired one oligonucleotide (carrying the iminobiotin moiety) with a library of 138 oligonucleotide derivatives, carrying different chemical groups (one of them being iminobiotin, with code number **76**). Additionally, we studied the selection of one oligonucleotide (carrying a cyanine dye as a chemical group with no detectable binding affinity for *streptavidin*), paired with the library of 138 compounds.

Both libraries were subjected to affinity selection experiments by using *streptavidin* immobilized on sepharose beads, either in the presence or in the absence of free iminobiotin as competitor. After several rounds of washing, the beads were used as a template for a modified polymerase chain reaction (PCR) amplifying the codes of the preferentially enriched library compounds. The fluorescence signals on the microarray, corresponding to the hybridization with the PCR product, revealed a preferential enrichment of the bidentate iminobiotin derivative (over the monodentate iminobiotin derivatives) in the first selection (Figure 4A), and of the monodentate iminobiotin derivative **76** in the second selection (Figure 4B).

For most practical applications, synergistic binding pairs coupled together by bifunctional chemical cross-linkers are more suitable than those scaffolded by heteroduplex DNA. Based on the results of the band-shift experiments with different pairs of iminobiotin-oligonucleotide conjugates, we selected Cy5, in which the exit vectors are similar both in distance and direction to those in the DNA heteroduplex, as a chemical scaffold (Figures 5A and 5B). We synthesized a bis-iminobiotin derivative of Cy5 with flexible linkers, and we determined its affinity to streptavidin by isothermal titration calorimetry. The bis-iminobiotin conjugate has a dissociation constant, K_d, of 6 nM to streptavidin, which corresponds to a >2000-fold affinity increase when compared to free iminobiotin, which displays a K_d of 13 μM (Figures 5C and 5D). The corresponding mono-iminobiotin conjugate did not display any binding under conditions similar to those for the bis-iminobiotin conjugate.

Significance

The results of this study have taught us valuable lessons for affinity selection experiments. First, bidentate ligands engaging adjacent epitopes on a target protein enjoy a strong affinity gain due to the chelate effect. Second, DNA appears to be an excellent scaffold for

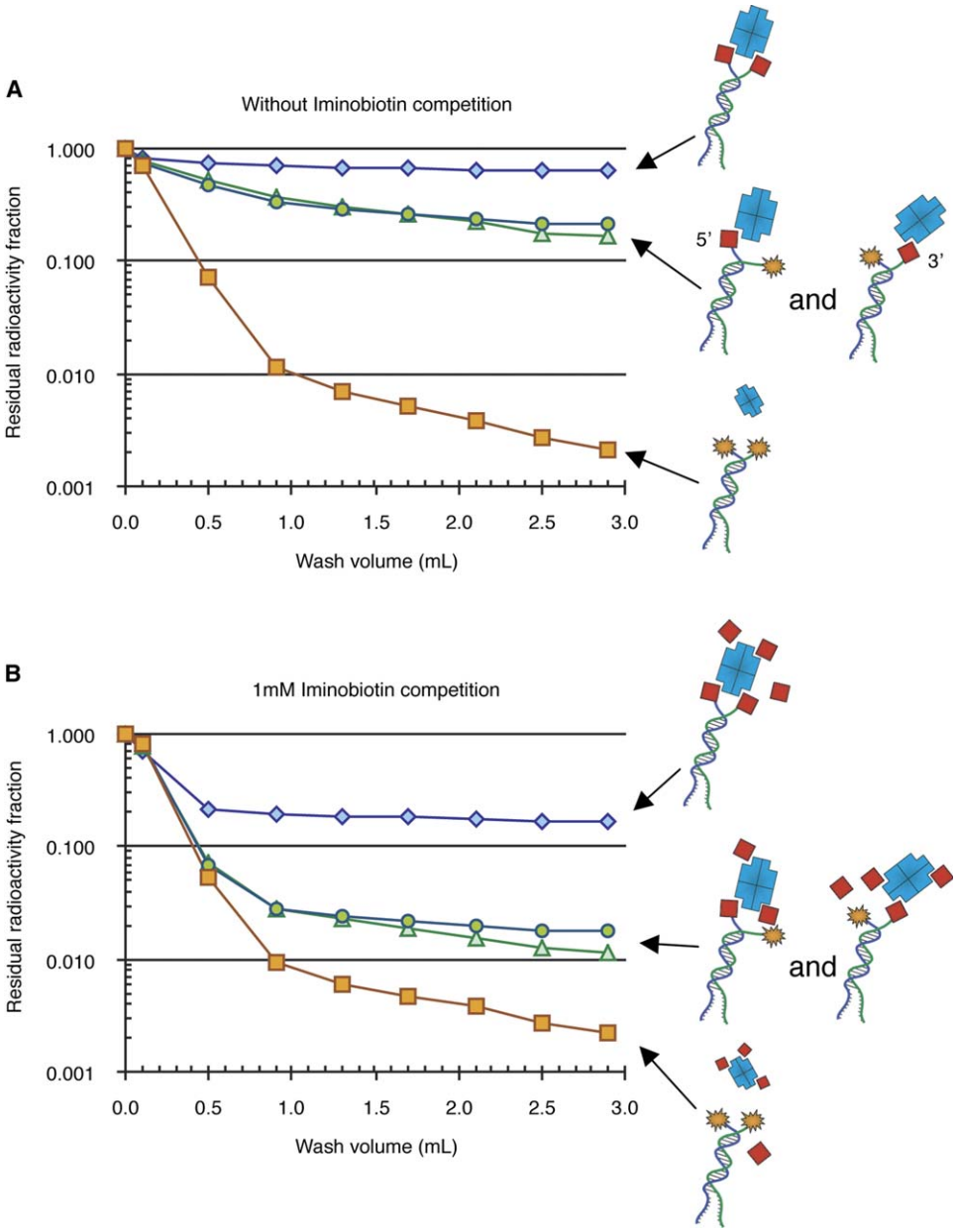

Figure 3. Recovery of Ligands in Affinity Selection Experiments

(A and B) Recovery profiles of bidentate and monodentate iminobiotin ligands, as well as of negative control ligands, in affinity selection experiments to *streptavidin*, as determined by using radiolabeled oligonucleotides. Aliquots of the different stages of the selection procedure were collected, subjected to radioactivity measurements, and normalized to the input value. The curves were drawn by plotting the fraction of radioactivity present in the eluate (last data point on the x axis), and by adding up the fractions of radioactivity measured in the different washing steps in the selection procedure. In order to alter the stringency of the affinity selection, the experiment was performed (A) without competition or (B) in the presence of 1 mM free iminobiotin as competitor.

synergistic binding moieties, but the choice of suitable exit vectors for the functional groups strongly influences the binding affinity. Third, high-affinity bidentate ligands are captured preferentially and with excellent recoveries, and these selections can be monitored not only with radioactive counting, but also with oligonucleotide microarrays, thus allowing the identification of preferred binders. Adjusting the stringency in the washing steps of the selection influences the relative recovery of ligands with different affinity for the targets. Fourth, it is possible to convert the selected pharmacophores to high-affinity bivalent binders by selecting suitable chemical scaffolds.

The combination of affinity selections [16, 17] and DNA encoding of chemical compounds [3–9] is facilitating the isolation of small organic molecules capable of specific protein recognition. Fragment-based identification of bidentate ligands [2] is a promising avenue for the development of general methods for disrupting protein-protein interactions, one of the big open challenges in medicinal chemistry and chemical biology.

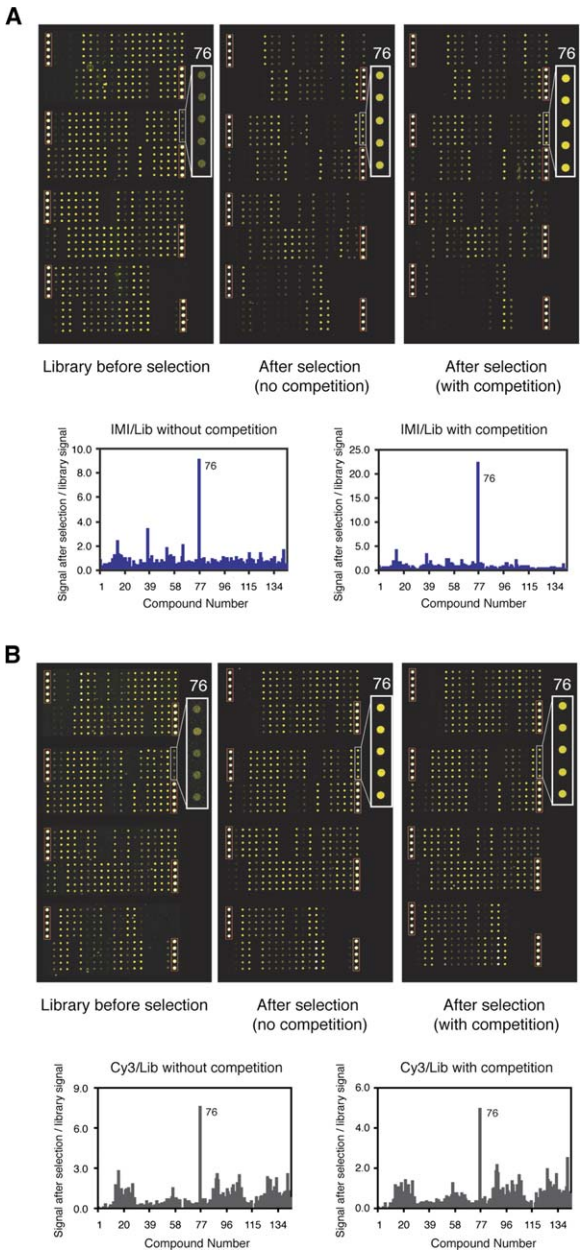

Figure 4. Readout of Selection Results by Microarray Technology

(A and B) Microarray decoding of test selection experiments against *streptavidin* by using a 138-compound library paired with an oligonucleotide covalently conjugated to (A) iminobiotin or (B) an irrelevant organic molecule (Cy3). The three microarray panels represent the microarray signals before selection, after selection without competition, and after selection with competition with 1 mM free iminobiotin. The five spots representing the signal for compound **76** (iminobiotin) are magnified, while the signals of positive control oligonucleotides are highlighted by red boxes. The graphs represent ratios of the average signal intensities for individual compounds after and before selection.

Experimental Procedures

Abbreviations

Abbreviations used in this paper are as follows: iminobiotin, 5-(7-amino-3-thia-6,8-diazabicyclo[3.3.0]oct-6-en-4-yl)pentanoic acid; NHS, N-hydroxysuccinimido ester derivative; TEA, Triethylamine.

Materials

Amino-modified and fluorescently labeled oligonucleotides were purchased from IBA (Göttingen, Germany). Oligonucleotide microarrays and the 137 compound library were produced as described [8]. Chemicals were purchased from Sigma-Aldrich-Fluka (Buchs, Switzerland), unless otherwise stated. The oligonucleotides used in band-shift assays have the following sequences (the hybridization sequences are underlined). Sequence of 5′ iminobiotin-modified oligonucleotides (with linker structures a, b, or c; Figure 1B): iminobiotin-(linker a/b/c)-5′-GGAGCTTCTGAATTCTGTGTGCTGGATAAACGACACGAATTCCGCAGC-(Cy3)-3′; sequence of 3′ iminobiotin-modified oligonucleotides (with linker structures x, y, or z; Figure 1B): 5′-CAGCACACAGAATTCAGAAGCTCC-3′-(linker x/y/z)-iminobiotin. For monodentate iminobiotin derivates, B3′(Cy3) (5′-CAGCACACAGAATTCAGAAGCTCC-3′-(z)-Cy3) or B5′(Cy3) (Cy3-(b)-5′-GGAGCTTCTGAATTCTGTGTGCTG-3′) were used as dummy pairing oligonucleotides. For quantitative model selection experiments, the following oligonucleotides were used: IMI(5′): 5′-iminobiotin-(b)-GGAGCTTCTGAATTCTGTGTGCTGATTGGCCGACACGAATTCCGCAGC-3′; IMI(3′): 5′-TCGCGAGGGGAATTCGTCATTTACCAGCACACAGAATTCAGAAGCTCC-(z)-iminobiotin-3′; Cy3(5′): 5′-Cy3-(b)-GGAGCTTCTGAATTCTGTGTGCTGGTGTGCCGACACGAATTCCGCAGC-3′; Cy3(3′): 5′-TCGCGAGGGGAATTCGTCGTTAAGCAGCACACAGAATTCAGAAGCTCC-(z)-Cy3-3′.

Chemical Conjugation of Iminobiotin to Oligonucleotides

Compounds were added to 120 μl of a 40% DMSO solution, reaching the following final concentrations: iminobiotin-NHS, 1.6 mM; amino-modified oligonucleotide, 85 μM; TEA/HCl buffer (pH 10), 400 mM. The coupling reactions were stirred overnight at room temperature, and residual activated species were then quenched by the addition of 50 μl 1 M Tris-Cl buffer (pH 8.0). The reaction was purified by HPLC on a XTerraRP C18(2) column (Waters, Milford, MA) by using a linear gradient from 100 mM triethylammonium acetate (TEAA) (pH 7.0) to 100 mM TEAA containing 80% (v/v) acetonitrile (pH 7.0). Solvents and volatile salt were removed under vacuum. The dried oligonucleotides were resuspended in 1 ml 20 mM TEA/HCl (pH 10) and were loaded on 0.5 ml avidin sepharose resin slurry (Affiland, Ans-Liege, Belgium). After washing the resin twice with 2 ml 20 mM TEA/HCl (pH 10), the iminobiotin oligonucleotides were eluted with 0.1% TFA (pH 2). After neutralizing the pH by addition of 1 M $NaHCO_3$, the iminobiotin oligonucleotide conjugates were again dried under vacuum.

Band-Shift Assays

The oligonucleotides carrying an iminobiotin moiety at the 5′ end were Cy3 labeled at the 3′ end, thus allowing for oligonucleotide detection on gels. Combinations of oligonucleotides were mixed in equimolar ratio with 150 mM Tris-HCl, 10 mM $MgCl_2$ (pH 8.9) as buffer, and they were incubated for 3 min at 94ºC and for 10 min at 50ºC to promote hybridization. Band-shift assays were performed by incubating the mono- and bidentate iminibiotin derivatives with various concentrations of *streptavidin* in 150 mM Tris-HCl, 10 mM $MgCl_2$ (pH 8.9) for 1 hr prior to loading onto a 15% native PAGE gel [18]. The final concentration of double-stranded oligonucleotide in the 10 μl of sample loaded on the gel was 6.6 nM. The final monomeric *streptavidin* concentrations were as follows: 103 μM, 34 μM, 11 μM, 3.8 μM, 1.3 μM, 420 nM, 140 nM, 47 nM, and 0 nM. The gels were run for 2 hr at 100 V. Detection was performed with a Diana III imager, using Cy3-filters (Raytest, Germany). The cage effect, i.e., the improved kinetic stability of complexes within the native polyacrylamide gel matrix, extends the scope of band-shift experiments for complexes with dissociation constants ranging from picomolar to micromolar values [12, 13].

Quantitative Model Selection Experiments

Oligonucleotides IMI(3′) and Cy5(3′) were radioactively labeled at their 5′ termini by using γ^{33}P-ATP (Amersham) and T4 polynucleotide kinase (USB). In four separate tubes, the four possible pairs of oligonucleotides were mixed at 45 nM concentration, and hybridization was allowed to occur as described above. To this solution, 50 μl *streptavidin*-sepharose slurry (GE Healthcare), which had previously been preblocked in 1 mg/ml herring sperm DNA, was added. After 30 min of incubation, free iminobiotin was added to an end

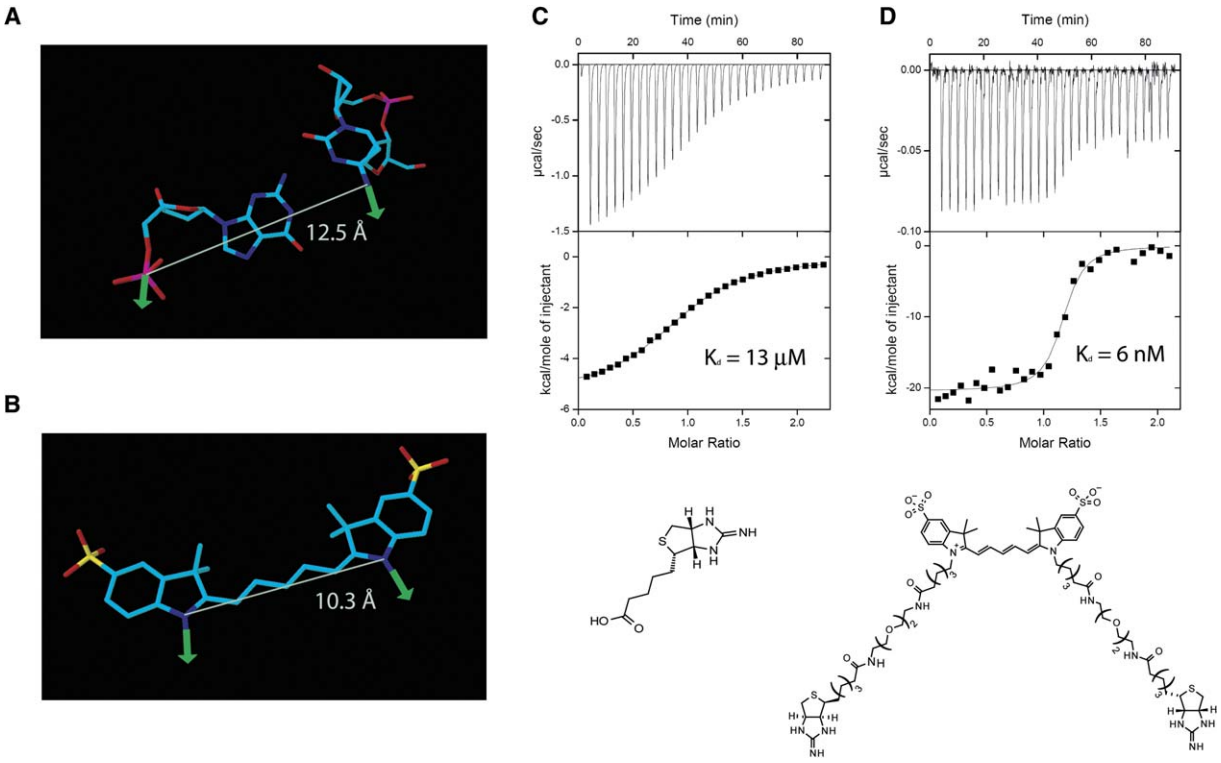

Figure 5. Bis-Iminobiotin Cy5 Conjugate Displays High Affinity to Streptavidin

(A) Three-dimensional model of the terminal bases and their exit vectors (arrows) of the DNA heteroduplex combination corresponding to the entities with the highest affinities to *streptavidin* in the band-shift experiment.

(B) Three-dimensional model of Cy5. The exit vectors are similar both in distance and direction to those in the DNA heteroduplex.

(C and D) Isothermal titration calorimetry profiles of the binding reaction of (C) free iminobiotin and (D) bis-iminobiotin to streptavidin reveal dissociation constants, K_d, of 13 µM and 6 nM, respectively.

concentration of 1 mM and was incubated for 5 min only in the competition experiments. After repeated rounds of washing, the remaining beads as well as aliquots of the input, flowthrough, and all washing fractions were subjected to ^{33}P radioactivity counting with a Beckman LS 6500 scintillation counter.

Test Selections with Library and Microarray Readout

Oligonucleotide with the code number **76** was coupled to iminobiotin as described above and were added to the previously described library of 137 compounds [8]. The resulting 138 compound library was paired with either oligonucleotide IMI(3′) or Cy3(3′). Selections with the resulting double-stranded libraries were performed with *streptavidin*-sepharose slurry as described above. For decoding, aliquots of the library before selection, and the samples after selection, were used as templates in a two-step PCR reaction (first step: 25 cycles of 1 min at 94ºC; 1 min at 55ºC; 20 s at 72ºC). In the first step, primers ABfo_short (5′-GGAGCTTCTGAATTCTGTGT-3′; 400 nM) and Aba (5′-GCTGCGGAATTCGTGTCG-3′; 400 nM; IBA) were used; however, in the second step, only the Cy3-labeled primer Aba (400 nM; IBA) was used. Additionally, in the second step, purified PCR products of the first step of the PCR reaction were used as templates (22 cycles of 1 min at 94ºC; 1 min at 55ºC; 20 s at 72ºC), yielding an excess of Cy3-labeled single-stranded target DNA. The reaction product was extracted with phenol/chloroform by using Phase Lock Gel tubes (Eppendorf, Hamburg, Germany) and was precipitated in ethanol. The DNA pellet was resuspended in 100 µl hybridization buffer (4× SSC, 50 mM HEPES, 0.2% [w/v] SDS [pH 7]) and was incubated with the microarrays in a Tecan HS4800 hybridization instrument (Tecan, Männedorf, Switzerland) for 4 hr at 44ºC; this was followed by successive washing steps with 2× SSC/0.2% (w/v) SDS, 0.2× SSC/0.2% (w/v) SDS, and 0.2× SSC for 90 s. After hybridization, microarrays were analyzed with a Scan Array 5000 instrument (Perkin Elmer; ex = 543 nm; 70% laser power; gain of photomultiplier = 90%). Spot intensities were quantified with GeneSpotter image analysis software (MicroDiscovery). The local background was substracted from the mean value of each spot, and the average of five spot values gave the signal value for each compound.

Synthesis of Cy5 Derivatives

1,8-diamino-3,6-dioxaoctane (400 µmol) and triethylamine (1 mmol) were mixed with 210 µl DMSO. Cy5 bis NHS ester (400 nmol, GE Healthcare) dissolved in 100 µl DMSO was added and stirred at 30ºC. After 12 hr, tris(hydroxymethyl)aminomethane (10 µmol) in 100 µl H_2O was added to quench unreacted NHS ester. The reaction mixture was purified on a Synergi 4u POLAR-RP column (Phenomenex, Torrance, CA) with a linear gradient from 0.1% TFA in H_2O to 100% acetonitrile. The fractions containing the mono- and bis-1,8-diamino-3,6-dioxaoctane-Cy5 derivatives were collected, and the solvent was removed. The Cy5 derivatives (~50 nmol) were redissolved in DMSO. Triethylamine (7.2 µmol) and iminobiotin NHS ester (1 µmol) dissolved in 100 µl DMSO were added. The reaction was stirred at 30ºC. After 12 hr, tris(hydroxymethyl)aminomethane (10 µmol) in 100 µl H_2O was added to quench unreacted NHS ester. The reaction mixture was purified on a Synergi 4u POLAR-RP column (Phenomenex, Torrance, CA) with a linear gradient from 0.1% TFA in H_2O to 100% acetonitrile. The fractions containing the mono- and bis-iminobiotin-1,8-diamino-3,6-dioxaoctane-Cy5 derivatives were collected, and the solvent was removed. The molecules were characterized by mass spectrometry.

Isothermal Titration Calorimetry

Isothermal titration calorimetry measurements were performed with a VP-ITC instrument (Microcal, Northampton, MA). 100 µM *streptavidin* was titrated with a 1 mM solution of iminobiotin. 1 µM Cy5 derivative was titrated with a 10 µM solution of *streptavidin* (after an

initial dummy injection of 1 μl, 29 injections of 10 μl each were performed). All solutions were 25 mM $NaHCO_3$, 150 mM NaCl, 5% DMSO (pH 11.1), and the experiments were performed at 30ºC. The resulting titration curves were then processed and fitted with the Origin 7 software.

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