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Directed Evolution of Streptavidin Variants Using In Vitro Compartmentalization

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SUMMARY

We have developed and implemented an in vitro compartmentalization (IVC) selection scheme for the identification of streptavidin (SA) variants with altered specificities for the biotin analog desthiobiotin. Wild-type SA and selected variants bind desthiobiotin with similar affinities ($\sim 10^{-13}$ M), but the variants have off rates almost 50 times slower and a half-life for dissociation of 24 hr at 25°C. The utility of streptavidin variants with altered specificities and kinetic properties was shown by constructing protein microarrays that could be used to differentially organize and immobilize DNAs bearing these ligands. The methods we have developed should prove to be generally useful for generating a variety of novel SA reagents and for evolving other extremely high-affinity protein:ligand couples.

INTRODUCTION

The interaction between streptavidin and biotin (dissociation constant of $\sim 10^{-14}$ M) is one of the strongest noncovalent interactions known in biology (Green, 1990; Hyre et al., 2006). Because of this, the streptavidin:biotin couple has been adapted to a huge number of applications, ranging from purification to immobilization to imaging methods.

Biophysical studies have provided a detailed molecular understanding of this high-affinity interaction (Stayton et al., 1999). In addition, the streptavidin:biotin couple has been reengineered, yielding designed streptavidin variants with altered affinities for biotin (Qureshi et al., 2001; Reznik et al., 1998), designed monomeric and dimeric streptavidins (Aslan et al., 2005; Qureshi et al., 2001; Sano et al., 1997; Wu and Wong, 2005), streptavidins with altered valency (Howarth et al., 2006), and alternatives to biotin as a ligand (Dixon et al., 2002; Hidalgo-Fernandez et al., 2006; Weber et al., 1994). Surprisingly, however, there have been few attempts to alter the couple using directed evolution (Aslan et al., 2005; Voss and Skerra, 1997).

In vitro compartmentalization (IVC) technologies have been developed as a means of linking proteins and protein phenotypes to the genes that encode them (Aharoni et al., 2005; Ghadessy et al., 2001, 2004; Griffiths and Tawfik, 2003; Mastrobattista et al., 2005; Sepp and Choo, 2005; Sepp et al., 2002; Tawfik and Griffiths, 1998). Water-in-oil emulsions contain femtoliter-sized aqueous reaction vessels in which transcription and translation of individual genes can be carried out. Because of the small size of the compartments, very large protein libraries (10^8–10^{12} variants) can be expressed, and sequence variants that lead to altered function can be captured by various selections and screens.

A number of variants of the basic IVC selection method have previously been used for the identification of affinity reagents. In one example, Sepp et al. (2002) showed that genes encoding peptides bearing a FLAG tag could be enriched by coimmobilizing the genes and an anti-FLAG antibody on a microbead. Following translation, compartments were lysed and bead-bound peptides (and their attendant genes) were fluorescently labeled and isolated by fluorescence-activated cell sorting (FACS). Using a different approach, Yanagawa and colleagues (Doi and Yanagawa, 1999; Yonezawa et al., 2003) showed the affinity enrichment of peptide-SA fusions and the biotinylated genes that encoded them. SA fusions translated within a compartment associated tightly with their genes. Once lysed, the peptides displayed on these synthetic phage were selected for the ability to bind either to a nickel affinity resin or to an anti-FLAG antibody. More recently, Bertschinger et al. (2007) employed a similar approach for the selection SH2 domains capable of binding mouse serum albumin with high nanomolar affinities. In this system, the SH2 library was fused to a methyl transferase and the dsDNA library tagged with suicide inhibitor of the methyltransferase such that upon translation, genotype and phenotype were linked covalently.

An alternate approach is to physically couple a ligand to the initial gene library so that translated gene products within individual compartments can directly associate with the genes that encode them. Those genes that produce the most functional proteins will best bind their tagged DNA templates, facilitating subsequent copurification, amplification, and selection. Unlike other methods, this approach relies solely on the off rate of the interaction formed within the compartment. Because the selected interaction is noncovalent, receptors can potentially have extremely high affinities. Sepp and Choo (2005) used a gene encoding zinc finger binding sites and a zinc finger binding protein known to have picomolar affinity for these sites to test this approach. Though this was successful, multiple zinc finger binding sites were used to increase binding through avidity, just as multiple receptors displayed on the surfaces of beads have mediated avid binding.

We have now implemented this method for the identification of extremely high-affinity interactions between individual receptors and ligands. We have expressed streptavidin variants in aqueous

compartments and identified those that can better bind genes coupled to a biotin analog, desthiobiotin. To ensure that we selected for extremely high-affinity interactions, genes were labeled with only a single ligand. Unlike the wild-type protein, the selected streptavidin variants bind both biotin and the analog desthiobiotin with similar affinities ($\sim 10^{-13}$ M). These altered binding properties may prove useful for the organization or assembly of nanostructures and arrays (Salem et al., 2004; Yan et al., 2003) or for the patterning of biomolecules on solid surfaces (Moll et al., 2002; Sabanayagam et al., 2000). The in vitro method not only allows for the rapid screening of very large libraries but also circumvents the innate toxicity of streptavidin (Szafranski et al., 1997) and yields variants with better folding properties. Such selection methods can also be used to augment our physicochemical understanding of the streptavidin-biotin couple and other high-affinity interactions.

RESULTS AND DISCUSSION

Selecting Streptavidin Variants via IVC

To generate streptavidin variants with altered ligand specificities we have used directed evolution within in vitro compartments. This method allowed us to examine relatively large library sizes (up to 10^{10} variants) and to finely control the process of selection. SA protein variants were coupled within each aqueous compartment to biotinylated genes encoding these variants (Figure 1). The SA protein variants also contained a His tag, and upon breaking, the emulsion protein:gene complexes could be readily purified and the successful genetic variants amplified. Multiple cycles of emulsification, translation, affinity purification, and amplification should lead to the identification of SA variants with particular kinetics or physicochemical properties.

Though functional streptavidin has previously been translated in vitro (Doi and Yanagawa, 1999; Yonezawa et al., 2003), we initially performed a series of control reactions to determine if His-tagged streptavidin could capture biotinylated genes relative to nonbiotinylated genes. Only in vitro translation reactions containing biotinylated streptavidin genes yielded appropriate-sized PCR products (see Figure S1 available online). When the same reactions were performed in a water-in-oil emulsion at a concentration of ~1 gene per aqueous compartment, only biotinylated genes led to PCR products (data not shown).

To show that functional SA variants could be selected, a small library was generated in which two critical positions in the gene, Asn23 and Ser27, which contact the ureido group of biotin (Klumb et al., 1998; Reznik et al., 1998; Figure 2A, red residues), were replaced with random sequence codons (NNN). The resultant library consisted of 4,096 gene variants, with the wild-type sequence expected to occur once in every ~340 DNA sequences (2/64 × 6/64).

The SA library was amplified using a 5′-biotinylated primer. After purification, 100 pg of the biotinylated library ($\sim 10^8$ variants) was emulsified along with an in vitro transcription translation (IVTT) reaction mixture. Under these conditions it was expected that only ~1 in 100 compartments should contain a single gene (with the bulk of the compartments containing no genes). The emulsified reaction was allowed to incubate at 30°C for 2 hr and then broken by extraction with water-saturated diethylether. To prevent functional gene products from associating with non-

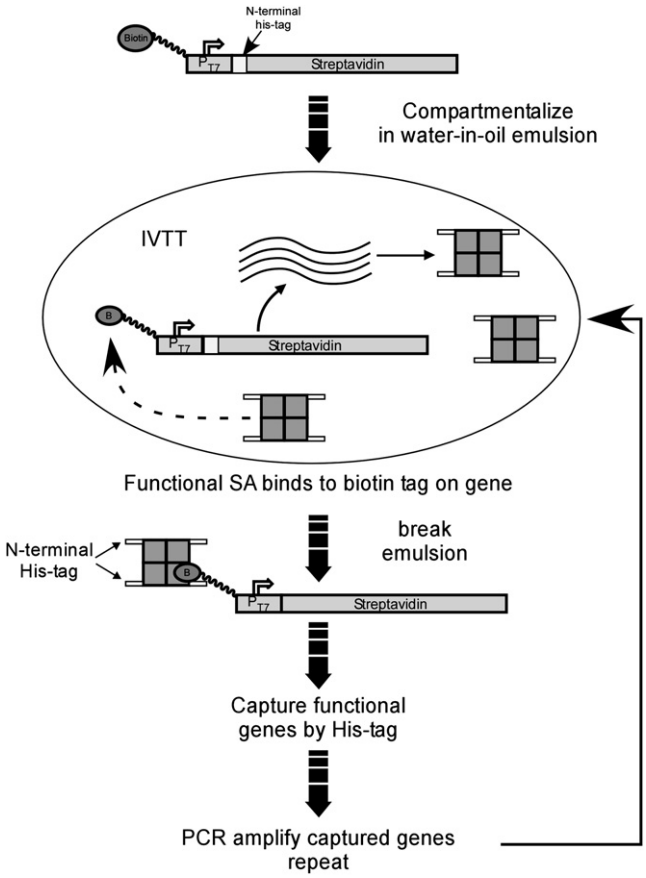

Figure 1. IVC Selection Scheme for Streptavidin Variants

Biotinylated genes encoding protein variants tagged with an N-terminal His-tag are compartmentalized within a water-in-oil emulsion along with the components of an in vitro transcription and translation reaction (IVTT). Functional proteins produced in the reaction associate with the genes that encoded them. Once the emulsion has been broken, the proteins are captured via their His-tags, and the copurified genes are amplified by PCR. The process can be repeated to select the most functional variants.

functional genes, the procedure was performed in the presence of a quenching buffer containing 100 μM free biotin. Protein products were captured on an anti-His antibody resin. After washing to remove unbound proteins and genes, captured products were eluted with 7 M urea and precipitated with ethanol. Recovered DNA was amplified with a 5′-biotinylated primer and used for an additional round of selection and amplification.

To determine whether selection was occurring, the initial and selected populations of genes were cloned and sequenced (Figure 3). Although the wild-type SA sequence did not appear in the unselected population (Figure 3, round 0), it comprised ~20% of the round 1 population and ~40% of population after round 2. Multiple, different Asn and Ser codons were found in the selected variants, suggesting that these were indigenous to the selection and not due to contaminating wild-type genes. The observed enrichment for a single round of selection was ~65-fold. This is similar to the level of enrichment observed by Sepp and Choo (2005) for the isolation of zinc finger proteins using a similar method, as well as for other protein selection technologies, such

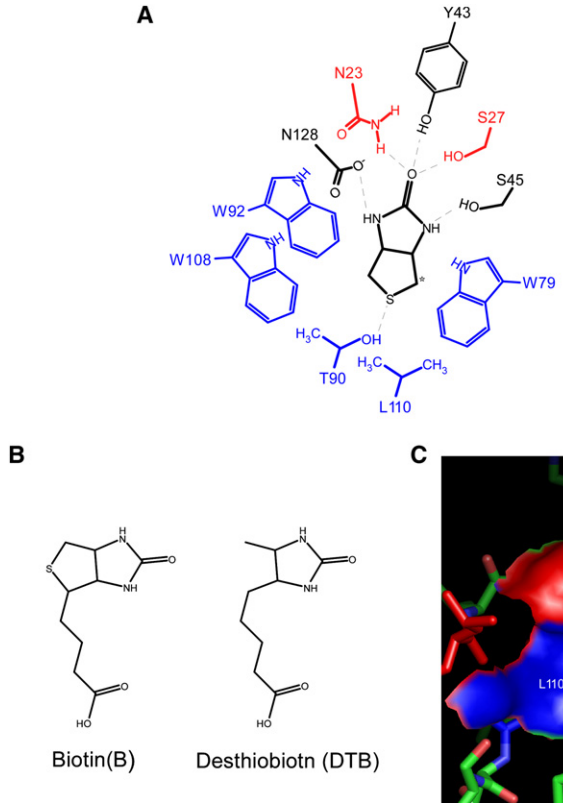

Figure 2. Pool Design

(A) Amino acid residues surrounding and contacting biotin in the streptavidin binding pocket (Weber et al., 1989). Residues that were mutagenized and selected for biotin binding are shown in red. Residues that were mutagenized and selected for desthiobiotin binding are shown in blue. Hydrogen bonds are represented by dashed lines.

(B) Structure of biotin and desthiobiotin.

(C) 3D representation of the positions of the biotin ureido and thiophene rings relative to mutagenized residues. Positions N23 and S27 are shown in white.

as phage display, *E. coli* surface display, and ribosome display (Gao et al., 1999; Hanes and Pluckthun, 1997; Jung et al., 2007). That similar enrichments are found by all of these methods further emphasizes the importance of plumbing very large library sizes to identify highly functional variants.

The selection also yielded a streptavidin variant that contained Ser at positions 23 and 27. Though this variant has not been previously reported, a functional assay showed that it is capable of binding biotin (Figure S2). Inspection of the streptavidin crystal structure suggests that the hydroxyl group of Ser23 may substitute for the amide group on the native Asn23 in forming a hydrogen bond to the ureido oxygen of biotin (Le Trong et al., 2003; Weber et al., 1989).

Having successfully tested the selection scheme using a small pool, we focused our attention on the design of larger pools for the selection of streptavidin variants with altered specificity. The biotin analog desthiobiotin binds to the streptavidin homolog avidin with a dissociation constant that is 10^2- to 10^4-fold weaker than that of biotin (Figure 2B; Green, 1970). Similar relative affinities are expected for streptavidin, where desthiobiotin has been shown to be readily displaced from the protein by the addition of biotin (Hirsch et al., 2002). Analysis of the crystal structure of streptavidin led us to hypothesize that five positions in the biotin-binding pocket formed contacts with the thiophene ring of biotin (Figure 2A, blue residues, and Figure 2C). In particular, W79, W92, and W108 all contribute to van der Waals/hyrdrophobic interactions, a major component of biotin's binding energy (Chilkoti and Stayton, 1995; Chilkoti et al., 1995; Freitag et al., 1998; Miyamoto and Kollman, 1993), whereas Thr90 has been reported to

Round 0

pos. 23		pos. 27	
AAT	Asn	AGC	Ser
ATC	Ile	CAT	His
ACT	Thr	ACT	Thr
AAA	Lys	CCC	Pro
CAG	Gln	GCT	Ala
ACA	Thr	ATG	Met
GGG	Gly	CAA	Gln
CCT	Pro	TTA	Leu
AAT	Asn	ATA	Ile
CAG	Gln	GTT	Val
GAG	Glu	CAG	Gln
CAG	Gln	TCG	Ser
AGA	Arg	TCT	Ser
TTT	Phe	TGC	Cys
ATT	Ile	AGC	Ser
GGC	Gly	GCT	Ala
CTC	Lue	ACT	Thr
ACT	Thr	AAA	Lys
CGT	Arg	ATC	Ile
CTG	Lue	ATC	Ile
GTC	Val	CGA	Arg

Round 1

pos. 23		pos. 27	
AAT	Asn	AGC	Ser
AAT	Asn	TCT	Ser
TAA	stop	ACG	Thr
AGC	Ser	TCA	Ser
CCT	Pro	ATT	Ile
AGC	Ser	TCT	Ser
TCT	Ser	AGT	Ser
ACA	Thr	TCA	Ser
AAT	Asn	AGT	Ser
CTA	Leu	TCG	Cys
TCA	Ser	TCT	Ser
AAT	Asn	AGC	Ser
AGC	Ser	TCA	Ser
ACT	Thr	AAA	Lys
GTA	Val	CAA	Gln
TCT	Ser	TCC	Ser
ATA	Ile	CCC	Pro

Round 2

pos. 23		pos. 27	
AAT	Asn	AGC	Ser
TCC	Ser	TCG	Ser
AAT	Asn	TCA	Ser
TCC	Ser	TCC	Ser
TCA	Ser	TCT	Ser
CTA	Leu	AAT	Asn
AAT	Asn	TCT	Ser
AGC	Ser	TCA	Ser
TCA	Ser	TCA	Ser
AAT	Asn	AGT	Ser
AAC	Asn	TTC	Phe
AAC	Asn	TCA	Ser
AAT	Asn	TCA	Ser
TCC	Ser	TCC	Ser
AAT	Asn	AGC	Ser
TCC	Ser	TCA	Ser
TCT	Ser	TCA	Ser

Figure 3. Progress of the Selection for Biotin Binding

The identities of sequenced variants at positions 23 and 27 within the streptavidin gene from round 0 (starting population), round 1, and round 2 are shown. Wild-type amino acids and codons are indicated at the top of each column. Selected proteins that contain the wild-type amino acid residues (Asn23 and Ser27) are shown in dark gray. The Ser23, Ser27 variant identified in this study is highlighted in light gray.

contact the sulfur in the thiophene ring (Weber et al., 1989). A library was therefore generated in which all five of these residues were completely randomized. To maximize the diversity of the library, the randomized positions were generated via oligonucleotides synthesized from trimer phosphoramidites (Kayushin et al., 1996). In addition to decreasing the total size of the library by more than an order of magnitude ($20^5 = 3.2 \times 10^6$ variants versus $32^5 = 3.4 \times 10^7$ variants), the use of trimer phosphoramidites eliminated nonfunctional nonsense variants from the library.

Following gene assembly, the double-stranded DNA (dsDNA) library was amplified with a primer bearing the biotin analog desthiobiotin at its 5′ end, and was used to initiate four iterative of rounds selection for desthiobiotin binding. The first two rounds of selection employed 300 pg of dsDNA corresponding to $\sim 3 \times 10^8$ sequences. Thus, every member of the population should have been present ~100 times. Emulsified translation reactions were incubated for 2 hr at 30°C, and the emulsion was then broken with diethyleither in the presence of 10 μM free desthiobiotin. Following affinity capture, the His-tagged gene products were eluted and PCR amplified, and the process was repeated. Rounds 3 and 4 were conducted using smaller amounts of pool DNA (30 pg for round 3 and 3 pg for round 4), and the incubation time was shortened to 1 hr at 30°C. This modification further ensured that individual compartments likely contained only a single gene, and that genes would not mix because of fusion between compartments. Additionally, in these later rounds, emulsions were broken in the presence of both 10 μM desthiobiotin and 100 μM biotin.

After four rounds of selection, the selected population was assayed for its ability to preferentially capture desthiobiotinylated DNAs. Briefly, double-stranded DNAs encoding either round 0, round 4, or the wild-type streptavidin gene were amplified with an unlabeled 5′ PCR primer. Following amplification and purification, the PCR products were added to an in vitro translation reaction. The reaction was allowed to proceed for 1 hr at 30°C, after which the reaction was terminated by the addition of the antibiotic kanamycin and split into three aliquots. A different reporter molecule was added to each aliquot, the round 0 population labeled with a 5′ biotin, 5′ desthiobiotin, or no tag at all. A portion of each binding reaction was then purified by affinity capture of the His tag, and the amount of DNA thereby recovered was determined by real-time PCR. As shown in Figure 4A, the round 4 population efficiently recovered genes labeled with either desthiobitin or biotin.

Sequence analysis of round 4 revealed that all members of the population shared the same amino acid substitutions within the randomized region: T90S, W108V, and L110T (Figure 4B). Additionally, all of the variants contained an additional two mutations located outside of the original randomized region: F29L and R53S (Figure 4B). One protein variant, R4-6, contained only these five substitutions and occurred in three of eight clones isolated from the round. Other variants contained both these consensus substitutions and some additional substitutions as well.

Given that mutations inside and outside of the randomized region seemed to be fixed during the selection, we decided to examine a larger number of sequence substitutions. The round 3 and round 4 populations were each mutagenized to a level of ~2.5% and ~5% via mutagenic PCR (as determined by DNA sequencing; data not shown). The mutagenized populations were pooled, and additional sequence diversity was introduced via DNA shuffling (Stemmer, 1994). The mutagenized and reassembled genes were then put through an additional three rounds of selection. Round 5 was carried out under conditions similar to those used for rounds 3 and 4. However, in rounds 6 and 7 the selection stringency was further increased by incubating the deemulsified reactions for 2 hr in the presence of additional competitors (10 μM biotinylated and desthiobiotinylated DNAs) prior to affinity capture. In this way, we ensured that selected SA variants would not merely develop the ability to interact nonspecifically with DNA.

Though the round 5 population showed no ability to bind desthiobiotinylated DNAs (as measured by real-time PCR assay), by round 7 the population could capture a desthiobiotinylated DNA as efficiently as the wild-type SA could capture a biotinylated DNA (Figure S3). Sequence analysis of the round 7 population revealed that many members of the population had retained the five consensus substitutions from round 4. In addition, the variants contained a number of additional amino acid substitutions, including a common S-to-G substitution at position 52 (Figure 4B).

Selected clones from round 7 were individually translated in vitro and assayed via DNA capture and real-time PCR. For these experiments the mutant proteins isolated from the selection were translated from plasmid DNA, and a biotin- or desthiobiotin-labeled round 0 population was used as a real-time PCR reporter. Following transfer of the SA:DNA complexes to a NiNTA-coated plate, the complexes were challenged with a mixture of 10 μM free desthiobiotin, 10 μM desthiobiotinylated oligonucleotide, 10 μM biotnylated oligonucleotide, and 100 μM free biotin; any remaining DNA reporter was eluted and amplified. As shown in Figure 4C, when the experiment was conducted using the wild-type protein and a desthiobitinylated reporter DNA, almost no DNA was recovered. In contrast, the selected variants proved capable of retaining almost all of the desthiobitinylated reporter DNA. The dominant variant from the round 7 population performed better than the dominant variant from round 4.

Based on these results, variants R7-2 and R7-6 were selected for additional analysis. Though these variants shared the same amino acid substitutions within the biotin-binding pocket, they differed in the mutations outside this region. In particular, clone R7-6 does not contain either the F29L or the R53S substitutions previously found to be common at the end of round 4. At least some of these additional mutations may be involved in the folding of streptavidin. When the various streptavidin variants are expressed in *E. coli*, the wild-type shows extremely poor solubility (<10% is found in the lysate, and most remains in inclusion bodies). In contrast, ~70% of R7-2 and ~50% of R7-6 are found in the soluble fraction (Figure S4). It seems likely that one of the constraints on streptavidin function in emulsions was folding following IVTT, and that in the absence of cellular chaperones, protein variants capable of folding more efficiently were highly favored during directed evolution. It is interesting that a completely in vitro evolutionary schema yields phenotypes that are favorable for cell-based protein production.

A

	reporter gene label		
Sample	no tag	B	DTB
wtSA	0 %	48 %	3 %
R0	0 %	0 %	0 %
R4	0 %	58 %	44 %

B

position		79	90	92	108	110	
frequency	WT	W	T	W	W	L	other positions
1	R4-2	W	S	W	V	T	T18A, Y22H, F29L, R53S
1	R4-5	W	S	W	V	T	F29L, R53S, T115I
3	R4-6	W	S	W	V	T	F29L, R53S
1	R4-7	W	S	W	V	T	F29L, R53S, D67G, A89V
1	R4-8	W	S	W	V	T	F29L, R53S, H127R
1	R4-9	W	S	W	V	T	F29L, R53S, V55A
1	R7-1	W	S	W	V	T	F29L, S52G, R53S, R84H, K121R
1	R7-2	W	S	W	V	T	F29L, S52G, R53S,
1	R7-3	W	S	W	V	T	F29L, S52G, R53S, S62C, T71A
1	R7-6	W	S	W	V	T	Y22F, S52G, A89V, H117R
1	R7-7	W	S	W	V	T	F29L, S52G, R53S
1	R7-8	W	S	W	V	T	L40S, V47G, S52G, R53S, R84H, K121R, T123I
1	R7-9	W	S	W	V	T	T32A, S52G, A89V, T111I
1	R7-10	W	S	W	V	T	F29L, A50T, S52G, R53S,V55A

C

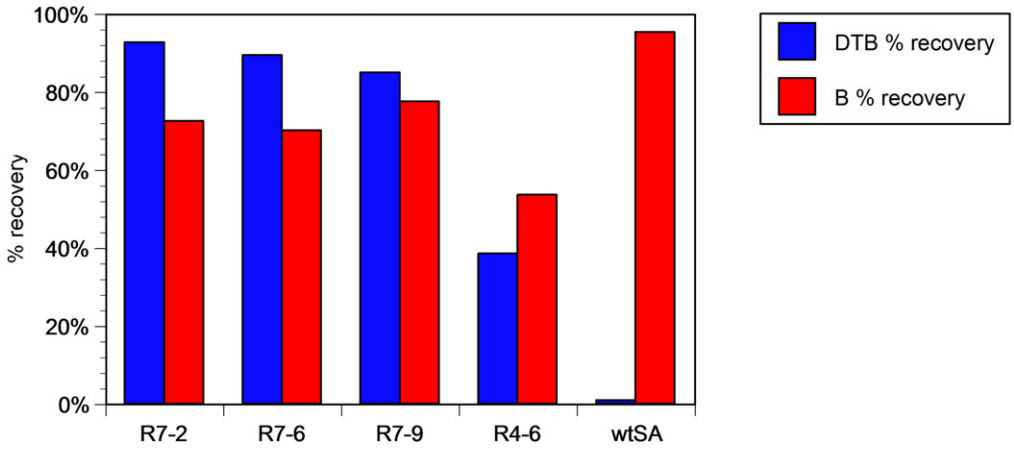

Figure 4. DNA Capture Assays with Selected Variants

(A) DNA capture assay of the round 0 (R0) and round 4 (R4) populations selected to bind desthiobiotin. IVTT reactions were supplemented with an unlabeled reporter gene (no tag), a biotinylated reporter gene (B), or a desthiobiotinylated reporter gene (DTB). The reported values represent the percent recovery on anti-His-tag antibody agarose resin of the reporter gene. wtSA is the wild-type strepavidin gene.

(B) Sequences of selected clones. Sequences from round 4 (yellow) and round 7 (orange) are shown. Residues that are common between the isolated variants are shown in red. The amino acid substitution S52G, which was common only to variants isolated from round 7, is shown in green.

(C) DNA capture assay of selected round 7 clones. IVTT reactions were supplemented with either a biotinylated reporter gene (B; red) or a desthiobiotinylated reporter gene (DTB; blue). After gene capture, protein variants were challenged with a mixture of competitors (see Experimental Procedures). The percent recovery was calculated from the ratio of the amount of reporter DNA recovered in the presence of competitor to the amount recovered in the absence of competitor.

Kinetic Characterization of Selected Streptavidin Variants

The rate constants for binding by SA variants were defined by measuring the off rate for either a ^{32}P-desthiobiotinylated oligonucleotide (DTB-T10), ^{32}P-biotinylated oligonucleotide (B-T10), or for free ^{3}H-biotin at 25°C. Rates were measured by preincubating proteins with trace amounts of radiolabeled ligand, followed by addition of an excess of cold ligand. Bound and free ligands were separated from one another by centrifugal filtration or $ZnSO_4$/NaOH precipitation (Klumb et al., 1998). As shown in

A

	DTB-T10	B-T10	biotin
pSA	2.0±0.15 (0.3)*	0.005±.0007 (137)	0.017±0.0002 (41)
wtSA	1.6 ±0.12 (0.4)*	0.004±.0005 (177)	0.016±0.002 (42)
R4-6	0.34 ±0.03 (2)†	0.28 ±0.02 (2)†	-
R7-2	0.049±0.006 (14)	0.056±0.003 (12)	2.7±0.12 (0.3)¥
R7-6	0.029±0.001 (24)	0.044±0.006 (16)	2.0±0.12 (0.3)¥

All rates are given in hr^{-1}. Half-lifes are shown in parenthesises and are in hr.
*Rate based on a signle timepoint.
†Rate based on single experiment.
¥Measured by $ZnSO_4$ precipitation

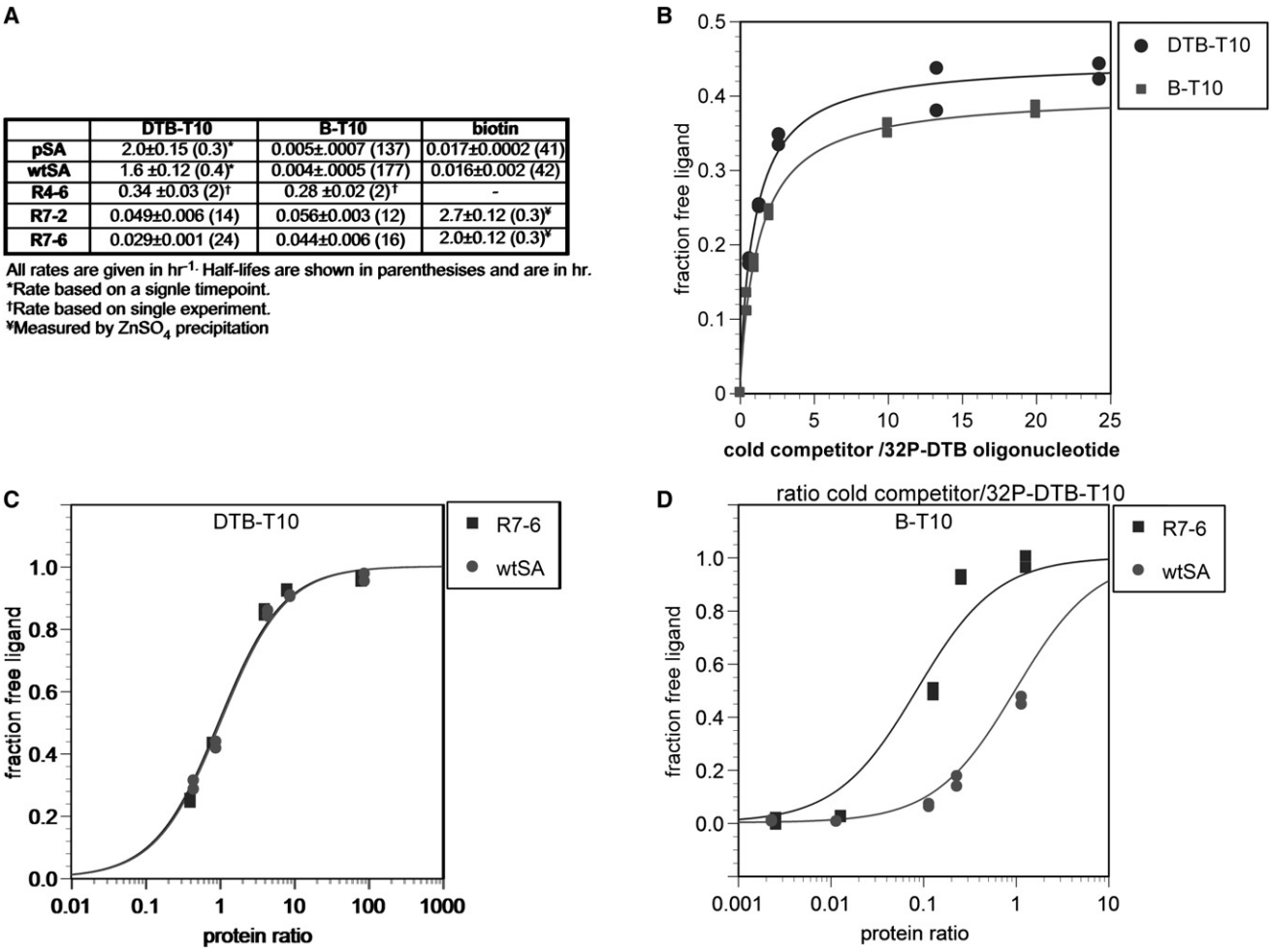

Figure 5. Kinetics of Streptavidin Variants

(A) Dissociation constants. Rates are given in units of $hours^{-1}$, with the half-life in hours for dissociation shown in parentheses. pSA is a commercial preparation of wild-type streptavidin. wtSA is wild-type streptavidin prepared in our lab. R4-6 is the dominant variant identified from round 4 and R7-2 and R7-6 are variants isolated from round 7.

(B) Oligonucleotide competition assay with protein R7-6 and trace ^{32}P-DTB-T10. The oligonucleotide competitors were either DTB-T10 or B-T10. The relative K_d values are reported in the text.

(C) Protein competition assay with ^{32}P-DTB-T10. Reactions contained a fixed concentration of either His-tagged R7-6 (squares) or His-tagged wild-type SA (circles), and increasing concentrations of nontagged, wild-type streptavidin (pSA). K_d values relative to wild-type streptavidin were 1.0 ± 0.1 in both cases.

(D) Protein competition assay with ^{32}P-B-T10. Reactions contained a fixed concentration of pSA and increasing concentrations of either His-tagged R7-6 (squares) or His-tagged wild-type SA (circles). Because no data points could be collected at higher ratios, the control experiment for the competition between pSA and His-tagged wild-type SA (red) was force fit with a relative K_d value of 1. For both (C) and (D), the protein ratio represents the concentration of pSA divided by the concentration of His-tagged R7-6 (squares) or His-tagged wild-type streptavidin (circles).

Figure 5A, both a commercially available streptavidin (pSA) and the His-tagged wild-type protein prepared in our lab (wtSA) release a desthiobiotinylated oligonucleotide quickly (a half-life of ~0.5 hr, compared with ~40 hr for biotin or ~150 hr for a biotinylated oligonucleotide). In contrast, clones R7-2 and R7-6 show much slower dissociation constants for the desthiobiotinylated oligonucleotide, with half-lives of 14 hr and 24 hr, respectively. This off rate approaches that of wild-type streptavidin with free biotin.

The changes in off rate were to be expected, as they were the focus of the selection experiments. Because the evolved variants displayed similar kinetics for the dissociation of both biotinylated and desthiobiotinylated oligonucleotides, we hypothesized that these substrates would be bound with equal affinities. To test this hypothesis we performed ligand-competition, equilibrium-binding experiments in which R7-6 was incubated with the ^{32}P-radiolabeled oligonucleotide DTB-T10 and increasing concentrations of the nonlabeled oligonucleotides DTB-T10 or B-T10 (Figure 5B). As expected based on the calculated off rates, both the desthiobiotinylated and biotinylated oligonucleotides yielded similar relative K_d values: 1.0 ± 0.1 for the competition with DTB-T10, and 1.3 ± 0.1 for the competition with B-T10. Thus, the selected variant shows only a slight equilibrium preference for desthiobiotin over biotin.

Based on these results, we also predicted that wild-type SA and variant R7-6 would have different affinities for desthiobiotinylated oligonucleotides because of their markedly different off rates. To test this hypothesis, a binding competition between different proteins (rather than different oligonucleotides) was carried out. The ^{32}P-radiolabeled DTB-T10 oligonucleotide was incubated with a fixed concentration of wild-type SA and increasing concentrations of R7-6. The two proteins showed almost identical affinities for the desthiobiotinylated oligonucleotide (Figure 5C). Consistent with these results, when a similar experiment was performed using a radiolabeled, biotinylated oligonucleotide (B-T10), the relative dissociation constant of R7-6 for biotin was calculated to be ~10-fold worse than that of the wild-type protein for biotin (Figure 5D). Because wild-type SA binds biotin with a K_d of 5×10^{-14} M (Green, 1990; Hyre et al., 2006), this implies that R7-6 binds biotin with a K_d of $\sim 10^{-13}$ M.

Because the dissociation constants of both proteins for DTB-T10 were similar, the observed ~50-fold decrease in the dissociation rate for desthiobiotin in the evolved R7-6 variant must be accompanied by a ~50-fold decrease in the association rate (again consistent with the fact that the selection was primarily for dissociation rate). The association rate for biotin and wild-type streptavidin is known to already be very fast ($\sim 1 \times 10^8$ $M^{-1}s^{-1}$; Hyre et al., 2006). Based on this rate, the estimated rate of association for our selected variant is $\sim 2 \times 10^6$ $M^{-1}s^{-1}$, similar to association constants previously reported for numerous monoclonal antibodies, including DNP-specific antibodies (3.4 and 9.5×10^6 $M^{-1}s^{-1}$; James and Tawfik, 2003), anti-hen egg lysozyme antibodies, (4.8×10^6 $M^{-1}s^{-1}$ at 25C; Xavier and Willson, 1998) and antifluorescein antibodies ($1.3–9.8 \times 10^6$ $M^{-1}s^{-1}$; Kranz et al., 1982).

These results imply that the simplest evolutionary route to increased discrimination between biotin and desthiobiotin does not involve simple changes in the thermodynamics of interaction due to changes in the binding pocket. Our original libraries were structured in part around this hypothesis: we expected that by changing the residues that directly contacted the relevant moieties on biotin and desthiobiotin we would achieve a discrimination based largely on interaction with these moieties and that would be reflected only in k_{off}. There is apparently no variant in the restricted space that we examined that could discriminate based only on contacting the thiophene ring. Instead, successful variants arose by accumulating additional mutations that altered the mechanism of desthiobiotin binding and recognition, so that both k_{on} and k_{off} were affected. A decrease in the association rate was also observed during the selection of a high-affinity antifluorescein single-chain antibody (Boder et al., 2000). It may be that the optimization of dissociation rate constants generally yields coupled changes in association rate constants.

Beyond the change in relative binding affinities for biotin and desthiobiotin, the selected variants also show a preference for oligonucleotide conjugates relative to free vitamin. Though the round 7 variants bind biotinylated oligonucleotides almost as well as the wild-type enzyme, they display a marked increase in the rate constant for the dissociation of free biotin ($k_{off} = 0.3$ hr).

Both round 7 variants show a significant improvement in off rate (~10-fold) over the dominant variant isolated from round 4, R4-6. In the case of R7-2 this is especially surprising, as this variant differs from R4-6 by only a single mutation, S52G. Analysis of the crystal structure shows that this mutation lies at the base of the flexible loop that forms the top of the biotin binding pocket. Structure studies of the wild-type protein have revealed that in the absence of biotin, this loop adopts an open conformation that is stabilized by a hydrogen bond formed between S52 and S45 (Freitag et al., 1997). Thus, the S52G mutation may directly affect the stability of this loop structure, and therefore the relative kinetics of binding to biotin and desthiobiotin. A selection for variants in the loop region also led to the isolation of streptavidin variants with improved affinities for a peptide ligand (Voss and Skerra, 1997). The selected substitutions were found to stabilize the loop in the open conformation (Korndorfer and Skerra, 2002). These directed evolution experiments, along with other biophysical and computational studies (Chilkoti and Stayton, 1995; DeChancie and Houk, 2007; Freitag et al., 1997; Hyre et al., 2006; Klumb et al., 1998; Miyamoto and Kollman, 1993), support a more dynamic model for biotin binding by streptavidin than is typically appreciated.

Utility of Streptavidin Variants with Altered Specificities

Streptavidin is one of the most widely used proteins in molecular biology, biotechnology, and most recently nanotechnology. The high stability and high affinity of the protein, coupled with the ease with which biotin can be functionally conjugated to other molecules, has led to the general use of this couple as a sort of molecular glue or Velcro. Streptavidin:biotin interactions have been used to organize biomolecules on solid surfaces (Bayer and Wilchek, 1990; Sabanayagam et al., 2000), in arrays (Peluso et al., 2003), and as components of designed nanoarchitectures (Cohen et al., 2008; Lee et al., 2005; Park et al., 2005; Yan et al., 2003). As an example of how streptavidin variants with different binding properties might be used to control biomolecular assembly, we constructed arrays of wild-type and R7-6 streptavidins, and used these to pattern biotinylated and desthiobiotinylated oligonucleotides.

Wild-type streptavidin (Figure 6, small spots) and the desthiobiotin-binding variant R7-6 (Figure 6, large spots) were alternatively spotted on an epoxy-activated glass slide. For each experiment a single array was subjected to two consecutive incubation steps. The first incubation involved a short duplex DNA labeled with either biotin and fluorescein, or desthiobiotin and fluorescein (Figures 6A and 6C). The second incubation was with a mixture of two duplex DNAs, one labeled with desthiobiotin and fluorescein, and the other with biotin and Cy5 (Figures 6B and 6D). After each incubation, the arrays were washed and imaged by fluorescent microscopy.

As expected, following the first incubation, both the wild-type protein (small spots) and the SA variant R7-6 (large spots) were fluorescently labeled with fluorescein, indicating that both of these proteins could bind to either biotin (Figure 6A) or desthiobiotin (Figure 6C). However, it was clear that the wild-type SA was less efficiently labeled with desthiobiotin (Figure 6C, small spots). This was likely due to its higher off rate for desthiobiotin and the partial removal of the desthiobiotinylated oligonucleotide by subsequent wash steps.

After imaging, the same protein arrays were incubated with the mixture of two duplex DNAs (Figures 6B and 6D). After a 2 hr incubation, the slides were washed and again imaged by

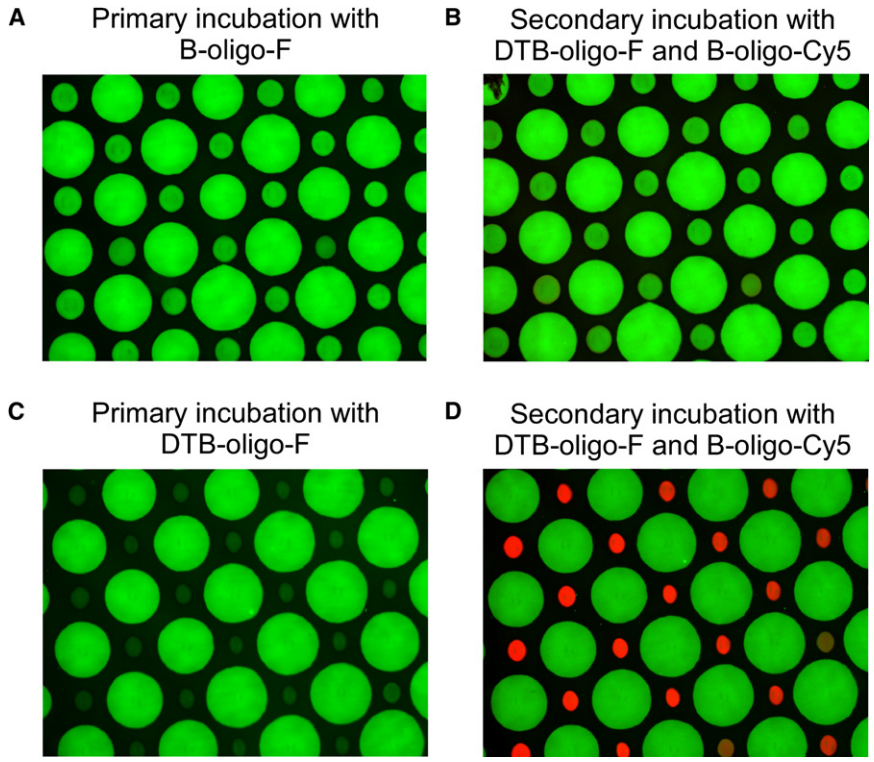

Figure 6. Dynamically Addressable Protein Arrays

(A–D) Wild-type streptavidin (small spots) and streptavidin variant R7-6 (large spots) were arrayed on epoxy-coated slides and first incubated with biotinylated (B) or desthiobiotinylated (DTB) oligonucleotides bearing fluorescein (F). The arrays were then challenged with a mixture of desthiobiotinylated, fluoresceinated, and biotinylated Cy5-labeled (Cy5) oligonucleotides.

microscopy. For the protein array that was originally charged with biotin, there was little change in signal. This was because the wild-type protein binds biotin more strongly than desthiobiotin, and both proteins have relatively slow off rates for biotin (Figure 6B). In contrast, for the protein array that was originally charged with desthiobiotin, the wild-type "changes its spots" and picks up the Cy5 label, whereas R7-6 does not (Figure 6D, small red spots). This reflects the fact that the wild-type streptavidin has a much higher off rate for desthiobiotin than does R7-6. Similar results were observed using the mutant R7-2 (Figure S5).

SIGNIFICANCE

We have used an IVC selection scheme to identify streptavidin variants with altered specificity toward the biotin analog desthiobiotin. Surprisingly, selected variants do not show an increased affinity for desthiobiotin when compared with the wild-type protein, but rather have altered binding kinetics such that both the off and on rates for this analog are ~50-fold slower than those of the wild-type protein.

The altered binding kinetics of our isolated streptavidin variants make them potentially useful tools for various biotechnological applications. Though desthiobiotin binds tightly to wild-type protein, it can be rapidly displaced by biotin. In contrast, our selected variants have high-affinity binding (~10^{13} M) for both biotin and desthiobiotin. Thus, the use of both proteins in parallel provides opportunities for discrimination based on their kinetic parameters. A structured protein array with the wild-type and selected streptavidins was labeled by the sequential addition of desthiobiotinylated and biotinylated substrates.

To the extent that the identity and patterns of immobilized ligands can be changed in a predictable way by simply changing the schedule of ligand applications, it may also prove possible to use streptavidin variants to create dynamically addressable nanostructures. Ligand additions would lead to a rational and predictable means of altering the compositions and structures of protein nanoarchitectures, similar to the way that oligonucleotide additions to metastable DNA structures have led to biomolecular devices capable of logic, sensing, and amplification (Shin and Pierce, 2004; Yurke et al., 2000; Zhang et al., 2007). New devices that use both programmable nucleic acids and programmable proteins might prove especially interesting. As a potential example, Milanovic and coworkers have developed multiarmed deoxyribozyme "spiders" that crawl across DNA surfaces (Pei et al., 2006). The spiders are organized by streptavidin "bodies," and streptavidin variants with altered kinetic properties now provide novel opportunities for coupling motion and self-organization.

Beyond the receptors produced, the selection method we describe is generalizable and can potentially be applied to various other small-molecule and protein interactions. Because of the requirement for long off rates ($t_{1/2}$ > 0.5 hr), the selection of extremely high-affinity interactions will be favored—an advantage over techniques that rely on covalent coupling or high avidity for capture.

EXPERIMENTAL PROCEDURES

Primers and Sequences

All oligonucleotides were purchased from IDT (Integrated DNA Technologies; Coralville, IA) with the exception of oligonucleotides containing a mixture of optimized codons, and biotinylated and desthiobiotinylated primers and substrates. For these latter oligonucleotides, synthesis was carried out in our laboratory on an Expedite 8909 DNA synthesizer (ABI; Foster City, CA) using standard phosphoramidite chemistry. The 5′ biotin was added using the biotin-TEG phosphoramidite and the 5′ desthiobiotin using the desthiobiotin-TEG phosphoramidite. Oligonucleotides containing a mixture of 20 optimized codons were generated using an equiactive mixture of phosphoramidite codons (trimer phosphoramidite mix 1, Glen Research; Sterling, VA). All synthesis reagents were purchased from Glen Research. Synthesis and deprotection was carried out using the manufacturers' protocols.

The wild-type streptavidin and streptavidin library used for the selection for desthiobiotin binding encompassed residues 13–140 of the mature full-length

protein (Sano et al., 1995) and were synthesized by PCR-based gene assembly from overlapping oligonucleotides. A complete list of the oligonucleotides used in this study, as well as the details regarding PCR-based gene assembly and mutagenic PCR, can be found in the Supplemental Data.

IVC Selection Scheme

The dsDNA library was added to 50 μl of an in vitro transcription and translation reaction (RTS 100, Roche; Indianapolis, IN) containing 0.5% sodium deoxycholate. All reagents were kept on ice. The reaction was emulsified by addition to 500 μl of an oil mixture containing mineral oil, 4.5% Span 80, 0.5% Tween 80, and 0.1% Triton X-100 (Ghadessy et al., 2001). The mixture was stirred for 4 min on ice, after which the emulsion was transferred to a 2.0 ml Eppendorf tube and incubated at 30°C. The amount of input dsDNA and the duration of the reaction for each round of selection are described in the text.

Emulsions were broken by 3 × 1 ml extractions with water-saturated diethyl ether in the presence of 500 μl of Tris-buffered saline (TBS) (100 mM Tris-HCl [pH 7.4], 150 mM NaCl) that also contained a quenching agent to prevent functional gene products from associating with nonfunctional genes. The quenching agent was free biotin, free desthiobiotin, a biotinylated oligonucleotide, a desthiobiotinylated oligonucleotide, or combinations thereof. The identity and quantity of quenching agent used for each round of selection are described in the text.

Following extraction, excess ether was removed by vacuum centrifugation for 5 min at room temperature. The remaining solution was added to 500 μl TBS with Tween 20 (TBST) (100 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing 100 μl of an anti-His antibody agarose resin (Sigma; St. Louis, MO). After a 30 min incubation, the resin was washed with 4 × 1 ml TBST and the captured products were eluted with 2 × 400 μl of 7 M urea mix (7 M urea buffered with 300 mM sodium acetate [pH 5.2]). Eluted fractions were combined and precipitated by the addition of 40 μg of glycogen and 2.5× vol of ethanol. The recovered DNA was PCR amplified using Hot Start Taq DNA Polymerase (Fermentas; Burlington, Ontario, Canada) and the primers RTS.F and RTS.R. The double-stranded PCR product was subsequently gel-purified (QIAGEN; Valencia, CA) and then reamplified using a labeled 5′ primer (5DTB.RTS.F or 5Bteg.RTS.F) and the standard reverse primer. Excess primers and nucleotides were removed using a PCR purification kit (QIAGEN), and the recovered DNA was used for subsequent rounds of selection. Individual selection rounds were cloned using the Topo TA cloning kit (PCR 2.1) (Invitrogen; Carlsbad, CA). Isolated clones were sequenced using the BigDye Terminator mix (Applied Biosystems; Foster City, CA) and analyzed on an AB3730 DNA Analyzer (Applied Biosystems).

Microarray Analysis of Streptavidin Variants

Protein arrays were generated using a custom-built robotic arrayer and pins designed to have a feature diameter of 100 microns (Majer Precision Engineering; Tempe, AZ). Proteins were diluted to 0.5 μg/μl in spotting buffer (100 mM NaHPO4 [pH 9.0], 50 mM NaCl and 50 mM trehalose, 5% glycerol) and were printed on epoxy-coated slides (SuperChip epoxysilane, Erie Scientific Company; Portsmouth, NH). Printed arrays were stored at 4°C without further processing for a minimum of 16 hr. The difference in spot sizes is likely the result of trace amounts of Tween 20 in the R7-6 preparation, which resulted in spreading of the spot.

Prior to each experiment, the spotted area of each slide was enclosed in a SIMplex 16 array holder (GenTel BioSciences; Madison, WI). The wells were blocked for 2 hr with 150 μl Protein Array Blocking Buffer (Whatman Schleicher and Schuell; Keene, NH). After blocking, the arrays were washed 3 × 150 μl of TBST. For a given array, the first incubation was performed with TBST containing a duplex DNA (100 nM) labeled with either biotin and fluorescein or desthiobiotin and fluorescein. The sequence and annealing conditions of the duplex oligonucleotides is provided in Supplemental Data. After the initial incubation, the arrays were washed with 3 × 150 μl of TBST and then imaged on an Olympus IX51 inverted microscope using FITC and CY5 filter sets. Exposure times were 1 and 5 s. Following imaging, the arrays were subjected to a second incubation with a mixture of two duplex DNAs (100 nM each), one labeled with desthiobiotin and fluorescein, and the other with biotin and Cy5. This second incubation was allowed to proceed for 2 hr, after which each well was washed with 3 × 150 μl of TBST and imaged by microscopy. Images were processed using ImageJ software (National Institutes of Health; Bethesda, MD). All images for a given filter set were thresholded to the same value.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Methods, Supplemental References, and seven figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/9/979/DC1/.

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