Dissecting the Streptavidin – Biotin Interaction by Phage-Displayed Shotgun Scanning

Sara K. Avrantinis, Ryan L. Stafford, Xia Tian, and Gregory A. Weiss*[a]

Shotgun scanning the streptavidin – biotin interaction identifies long-range hydrophobic interactions that contribute to one of the strongest naturally occurring noncovalent protein – ligand interactions. The femtomolar dissociation constant for this interaction makes it a useful model system to dissect the forces that govern high-affinity molecular recognition between proteins and small molecules. Shotgun scanning combines the diversity and in vitro binding selection of phage-displayed libraries with a binomial mutagenesis strategy. Libraries consist of proteins with the residues in multiple positions mutated to give a 1:1 ratio of alanine:wild type. Here, we use shotgun scanning to determine the functional contribution of the 38 C-terminal residues of streptavidin to the high-affinity interaction with biotin. The library pools were subjected to three rounds of selection for functional streptavidin variants that bind biotin and statistical analysis was used to assess side-chain contributions to biotin binding. The results demonstrate the utility of shotgun scanning for the dissection of receptor – small-molecule interactions. While shotgun scanning results were largely consistent with previous single-point, site-directed mutagenesis studies for residues in direct contact with biotin, residues distant from the biotin binding site have not previously been explored. Key streptavidin residues identified by shotgun scanning as contributors to the interaction with biotin include those with side chains that fill the β barrel, residues at the tetramer interface, and second-sphere residues, which are reinforced by long-distance propagation of hydrophobic interactions.

KEYWORDS:

mutagenesis · phage display · protein engineering · shotgun scanning · structure – activity relationships

Introduction

Streptavidin, a tetrameric protein isolated from *Streptomyces avidinii*, binds the small molecule biotin with extraordinary affinity (dissociation constant, $K_D = 4 \times 10^{-14}$ M).[1] The strength and reliability of the streptavidin – biotin interaction underlie its importance for biotechnology applications and as a model for high-affinity receptor – ligand interactions. These facts prompted us to choose the streptavidin – biotin system to demonstrate the utility of shotgun scanning for high-throughput investigations of receptor – small-molecule interactions. We report here for the first time systematic shotgun scanning of a receptor – small-molecule interaction to assess the side-chain contributions from 32% of core streptavidin residues to the interaction with biotin.

X-ray crystal structures,[2, 3] single-point site-directed mutagenesis experiments,[4–8] and circularly permuted streptavidin variants[9] suggest three binding site features critical to the strength of the streptavidin – biotin interaction. First, hydrophobic and van der Waals forces, primarily between biotin and four tryptophan residues, contribute key contacts. Second, a network of hydrogen bonds between streptavidin and the urea, thioether, and carboxy functional groups of biotin stabilize biotin binding. Additionally, a flexible streptavidin surface loop (residues 45 – 50) moves from an open conformation in the apoprotein to a closed conformation upon complexation with biotin.[2, 3] A detailed description of the role played by residues outside the biotin binding site is missing from our understanding of this canonically strong interaction.

Previous studies of the streptavidin – biotin interaction have focused exclusively on residues in direct contact with biotin, but other streptavidin residues could provide key supporting roles to the high-affinity binding of biotin. For example, "second-sphere residues," which neighbor the active site,[10, 11] could help orient directly contacting side chains. Long-range networks of hydrophobic residues can also position active-site residues. For example, the hydrophobic residues that compose the core of PDZ domains have been implicated in long-range energy conduction.[12] In addition, residues critical to protein folding are essential for providing of the framework for strong noncovalent interactions, but are not necessarily located in the binding site. The shotgun scanning method applied here can identify residues that contribute to protein functionality through both direct and indirect interactions.

Shotgun scanning applies diverse protein libraries in which combinations of residues in specific positions are varied between alanine and the wild-type (nonmutant) residue. Alanine substitutions, which remove atoms past the β-carbon, assess the

[a] *Prof. G. A. Weiss, S. K. Avrantinis, R. L. Stafford, X. Tian*
Department of Chemistry
516 Rowland Hall, University of California
Irvine, CA 92697-2025 (USA)
Fax: (+1) 949-824-8571
E-mail: gweiss@uci.edu

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importance of each side chain to protein function. The library pool is displayed on filamentous phage particles and in vitro selections are used to isolate library members that bind to an immobilized target ligand. Individual selectants are then screened for binding before sequencing. The occurrence of the wild-type or an alanine residue at each position is used to calculate the ratio of wild-type to alanine (wt:A). The wt:A ratio correlates with the contribution of a given side chain to ligand binding, such that the wt:A ratio for large, favorable contributions approaches n (where n is the total number of clones sequenced for each library); the wt:A ratio for a residue with a negative impact on binding approaches zero.[13]

Here, streptavidin shotgun scanning focused upon the 38 C-terminal positions (residues 96 – 133) of phage-displayed, tetrameric streptavidin.[14] These residues were chosen because they comprise several strands of the β-barrel structure and are located at the tetramer interface of streptavidin. Selection for binding to biotin was used to isolate functional mutants. Streptavidin provides an attractive system for the study of high-affinity interactions by this method because X-ray crystal structures of alanine-substituted streptavidin have revealed little structural perturbation as a result of this mutation of the protein.[6]

Results

Library construction

The streptavidin shotgun scanning libraries were constructed as described previously.[13] Briefly, degenerate oligonucleotides encoded a 1:1 ratio of alanine to wild-type amino acids in specific positions. Up to two other codons that resulted from degeneracy in the genetic code were also present in some positions (m2, m3, Table 1). Positions with wild-type alanine or glycine residues were not mutated (not shown in Table 1). Mutation of more than 30 residues in a single library would result in diversity that exceeds the capabilities of phage display. Therefore, the 38 C-terminal residues of the core of streptavidin (residues 96 – 133) were divided into two shotgun scanning libraries, each with a theoretical diversity of approximately 10^8 protein variants. The initial (naïve) libraries had diversities approaching the theoretical values. Sequencing of the naïve libraries confirmed that library construction resulted in a wild-type to alanine ratio of approximately 1:1. Functional, tetrameric streptavidin was displayed on the phage surface by positioning an amber stop codon between the open reading frames that encode streptavidin and P8. In an amber suppressor *Escherischia coli* strain such as XL1-Blue, the production of both free and P8-fused streptavidin monomers results in periplasmic assembly of the streptavidin tetramer.[15]

Formation of tetrameric, rather than monomeric, streptavidin was investigated by mutation of the amber stop codon to a serine codon and conduction of binding assays with both types of phage. The serine mutant phage exhibited significantly decreased biotin binding compared to the amber-stop-containing phage in binding assays (data not shown). Monomeric streptavidin binds biotin with a K_D value approximately five orders of magnitude higher than that of tetrameric streptavidin.[16] Therefore, the substantially reduced biotin binding capability of the serine mutant phage supports the hypothesis that tetrameric streptavidin is displayed on the phage surface.

Table 1. *Ratio of wild-type or mutated (m2, m3) residues to A, as recorded by streptavidin shotgun scanning.*[a]

Library 1 ($n = 105$)				Library 2 ($n = 83$)			
wt	wt:A	m2:A	m3:A	wt	wt:A	m2:A	m3:A
Y96	27.3	3.0 (S)	2.7 (D)	E116	1.3	–	–
V97	9.3	–	–	N118	2.9	1.8 (D)	1.3 (T)
E101	2.6	–	–	W120	3.0	1.3 (G)	0.2 (S)
R103	14.2	1.8 (G)	0.2 (P)	K121	2.9	1.1 (T)	0.2 (E)
I104	13.8	1.7 (V)	0.5 (T)	S122	3.3	–	–
N105	4.4	14 (D)	1.4 (T)	T123	2.6	–	–
T106	1.4	–	–	L124	7.7	2.7 (V)	0.3 (P)
Q107	0.3	0.1 (E)	0.1 (P)	V125	5.9	–	–
W108	21.0	2.8 (G)	1.3 (S)	H127	8.7	1.0 (P)	0.9 (D)
L109	2.4	6.8 (V)	0.1 (P)	D128	5.1	–	–
L110	10.3	1.4 (V)	0.3 (P)	T129	3.9	–	–
T111	2.1	–	–	F130	16.0	1.8 (V)	1.5 (S)
S112	4.1	–	–	T131	5.9	–	–
T114	1.7	–	–	K132	8.7	1.6 (E)	0.1 (T)
T115	0.9	–	–	V133	4.4	–	–

[a] Hyphens denote positions with only wt or A residues. Single-letter amino acid codes in parentheses designate the m2 or m3 substitution.

Selection of biotin-binding clones

The streptavidin libraries were selected for binding to biotinylated bovine serum albumin (BSA) immobilized on microtiter plates. Bound phage that displayed functional streptavidin were eluted under optimized conditions. After three rounds of selection, individual clones were grown in 96-well format and culture supernatants, which contained phage-displayed streptavidin mutants, were directly assayed by phage ELISAs[17] to identify streptavidin variants that retained affinity for biotin. Clones identified from the screen as biotin binders, 105 from library one (residues 96 – 115) and 83 from library two (residues 116 – 133), were sequenced and the wt:A ratio for each position was calculated (Table 1).

Wild-type streptavidin accounted for about 8% of the selected clones overall. Silent mutations encoded by the library oligonucleotides demonstrated that these wild-type selectants were from the library (that is, they were not contaminating phage that displayed wild-type streptavidin and did not result from the library template). This enrichment of the wild-type protein from two different libraries, each with diversities approaching 10^8 streptavidin variants, suggests that the selection conditions successfully identified high-affinity biotin binders. Furthermore, since library members competed with wild-type streptavidin during library selection, we expect the other functional clones to bind biotin with on (k_{on}) and off (k_{off}) rates comparable to those of the wild-type protein. A fairly high rate of identical sequences (siblings) was observed. The most common sibling composed 17% of the selectants from library 2; overall, approximately half of the sequences had at least one sibling.

Discussion

Caveats to streptavidin shotgun scanning

Shotgun scanning offers a high-throughput method for analysis of large numbers of protein residues, but several caveats need to be considered. First, the method implemented here requires growth of a potentially toxic protein, streptavidin, in *E. coli*. We have not encountered difficulties in growing streptavidin fused to the phage surface and others have reported high expression levels of wild-type streptavidin in *E. coli*.[18] Second, bound phage were eluted by vigorous shaking in 100 mM hydrochloric acid during rounds of selection for binding to the ligand. These elution conditions could select for mutations that enhance the sensitivity of the protein to low pH conditions. Furthermore, such conditions could paradoxically favor mutations that destabilize the tertiary and quaternary structures of streptavidin, which are both required for binding to biotin. The in vivo amplification step could also provide additional selection criteria; for example, proteolysis during growth in *E. coli* could select against unfolded mutants. Specific examples where these caveats to the technique could be important are noted in the discussion of our data. The high frequency of wild-type streptavidin selected from the diverse initial libraries indicates, however, that the growth, selection, and elution conditions described here identify high-affinity, properly folded, tetrameric streptavidin variants.

Streptavidin residues in direct contact with biotin

Previous studies of the streptavidin – biotin interaction have focused on a few residues in direct contact with biotin. Our streptavidin shotgun scanning data intersects with conventional biochemical studies in three positions, which can be used to calibrate the combinatorial mutagenesis described here. For example, W108 lines the biotin binding site and single-point, site-directed mutagenesis has demonstrated the importance of the W108 indole functionality for biotin binding.[4–6] Shotgun scanning analysis (Figure 1) confirmed a strong preference for tryptophan at residue 108 (wt:A = 21:1). In addition, the ureido group of biotin participates in a hydrogen bond network, anchored by a hydrogen bond from the carboxy group of D128.[2] In our study, D128 was preferred over alanine by a ratio of 5:1. Interestingly, in the case of W120, which has previously been shown to contribute significantly to biotin binding,[4–7] only a modest preference for the wild-type W residue was exhibited in these experiments (wt:A = 3:1). The W120A streptavidin mutant remains fully folded and tetrameric,[6] however, we cannot rule out the possibility that mutations in this position destabilize either the tertiary or quaternary structure of streptavidin. Such destabilization by this residue could facilitate elution after selection for binding to biotin, which would artificially depress the wt:A ratio. However, good agreement between previous studies and shotgun scanning for other residues suggests that shotgun scanning effectively identifies critical side-chain contributions to binding between proteins and small molecules and includes positions that have not been examined previously. For example, a strong preference for wild-type was observed at L110 (wt:A = 10:1), a position in close proximity to the valeryl functionality of biotin. The wt:A ratios could reflect relative contributions to biotin binding by specific side chains.

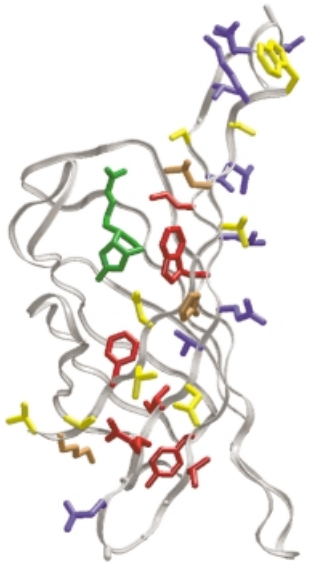

Figure 1. *Results from shotgun scanning. Side chains examined in this study (stick models) comprise 32% (residues 96 – 133) of streptavidin (gray). Colors indicate the ratio wt:A reported in Table 1. Red denotes wt:A > 9; orange, 9 > wt:A > 6; yellow, 6 > wt:A > 3; blue, wt:A < 3. Green represents biotin. Figures were produced by using Visual Molecular Dynamics[27] and Raster3D software.[28] (PDB accession code: 1STP[2]).*

Previous shotgun scanning studies have used wt:A ratios to calculate $\Delta\Delta G$ values and thus quantify the energetic contribution of individual side-chain functionalities.[13, 19] Typically, the ratio wt:A is treated as equal to the ratio of binding equilibrium constants for the wild-type and Ala mutant (K_{wt}:K_{Ala}). This ratio of equilibrium constants is then converted to the $\Delta\Delta G$ value that compares the wild-type to the Ala mutant ($\Delta\Delta G_{Ala\text{-}wt}$). Streptavidin, however, has an unusually slow off rate, with a half-life of about 3 days at 25 °C.[1] We cannot be certain that thermodynamic equilibrium was reached under the conditions used for library selection and therefore do not assume that the wt:A ratio is equal to the ratio of equilibrium constants for the wild-type and alanine-substituted mutant, respectively. Other streptavidin mutagenesis studies have shown that streptavidin residues whose mutation leads to large $\Delta\Delta G_{mut\text{-}wt}$ values effect off rates almost exclusively, while those with modest $\Delta\Delta$G values effect both k_{off} and k_{on} values.[4] Thus, we expect large wt:A ratios observed by shotgun scanning to mainly effect k_{off} values. Alanine substitutions in these positions are expected to result in faster off rates. This analysis, which focuses upon large ratios of wt:A, is consistent with previous shotgun scanning studies that demonstrated good correlation between shotgun scanning and conventional $\Delta\Delta G$ values above about 1 kcal mol^{-1}.[13]

Indirect contributions to biotin binding

In addition to highlighting the contributions of streptavidin residues in direct contact with biotin (W108, L110, D128), our

experiments also identified residues some distance from the binding site that contribute to the interaction, but have not previously been identified as contributors (Figure 1). Two stretches of highly conserved residues that extend across the β strands were revealed near the top and bottom of the streptavidin β barrel. These mainly hydrophobic residues are most likely responsible for β-barrel formation through hydrophobic collapse and interstrand cross-links from side-chain interactions. Residues unimportant for binding to biotin form the β turns at the ends of the strands. An understanding of the forces important for β-sheet formation is critical for design of β-sheet folds.[20–22] In the examined residues of streptavidin, contacts between neighboring interstrand residues, not from key residues at turns, stabilize β-barrel formation. A possible exception to the importance of interstrand side-chain cross-links is the case of R103, a nonburied residue highly conserved in these studies. R103 features a hydrogen bond that extends from the η nitrogen atom of the guanidine moiety back to its own β strand and has no obvious interstrand contacts. At the positions studied, the wild-type residue was strongly preferred and selection for mutants that destabilize the structure of streptavidin, possibly as a result of alanine substitutions, was not observed. We conclude that these residues contribute indirectly to biotin binding through stabilization of the streptavidin scaffold or other effects.

Results from shotgun scanning corroborate the expectation based on computational studies that interactions with hydrophobic side chains dominate the streptavidin – biotin interaction.[23] Every hydrophobic residue had a wt:A ratio greater than three, which demonstrates the importance of a hydrophobe-packed, rather than alanine-substituted, core for functional streptavidin. In fact, hydrophobic residues far from the binding site (Y96, F130) where shown to be as important to biotin binding as residues in direct contact with biotin. Therefore, the femtomolar streptavidin – biotin dissociation constant appears to be achieved by an exquisite organization of extended hydrophobic interactions that buttress residues in direct contact with biotin. We also find a fairly diffuse binding site with a large number of roughly equal side-chain contributions.

Surprisingly, Y96 and F130 were as critical to biotin binding as W108, a hydrophobic residue in direct contact with biotin. Y96 participates in the interstrand contacts described above. F130 appears to orient D128 for H bonding to the urea nitrogen atom of biotin and could provide assistance to binding as a second-sphere residue. Y96, W108, and F130 point into the β barrel and most likely assist protein folding. These residues could also participate in a network of side chains with interlocked hydrophobic interactions. Previous studies of streptavidin mutants by X-ray crystallography found that aromatic ring systems shifted slightly in the binding site, a finding that "point[s] to a more extended network of aromatic residues beyond the ones directly in contact with biotin."[24] Our study identifies two contributors to long-range hydrophobic networks as Y96 and F130. Further experiments are necessary to assign a precise role to these residues. In addition, shotgun scanning of other proteins could assess whether the interlocking hydrophobic interactions identified here are unique to the high-affinity streptavidin – biotin interaction.

Residues at the subunit interface

Based on the streptavidin crystal structure, W120 was expected to mediate formation of the streptavidin tetramer. The W120A mutant protein, however, remains tetrameric.[6] Here, we identify two additional residues that contribute to biotin binding at the subunit interface (Figure 2). The streptavidin monomers are arranged as a pair of dimers, with the dimers sharing hydrogen bonds and exchanging W120 residues.[2] In our study, H127 is conserved 9:1 over alanine and reaches across the dimer – dimer interface to interact with H127 from a neighboring subunit through an apparent imidazole – imidazole π-stacking or π-cation interaction. In addition, the V125 residues from each of the four subunits interact at the dimer interface with moderate importance to biotin binding (wt:A = 6:1).

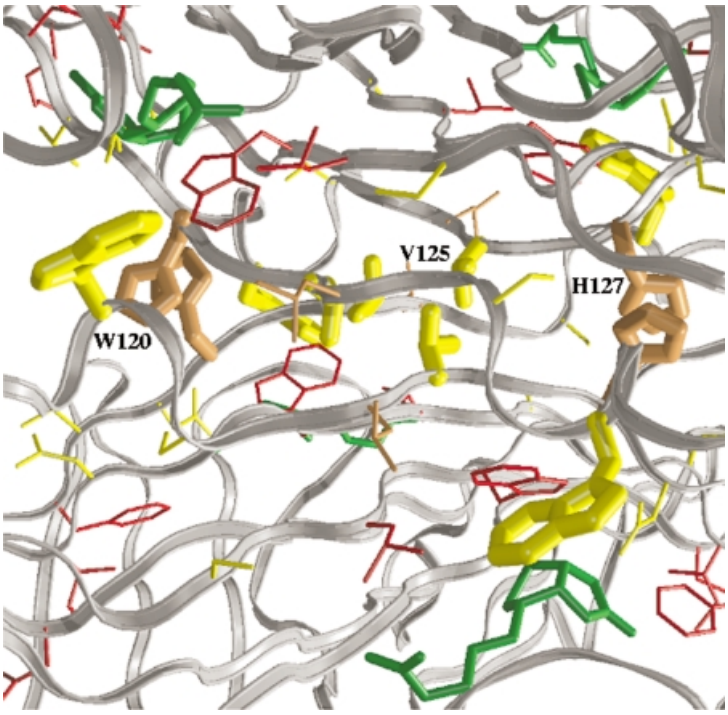

Figure 2. *Key residues at the streptavidin tetramer interface. Colors are as described for Figure 1. (PDB accession code 1SWE[29]).*

A preference for substitutions of residues different from the wild-type was observed for three streptavidin residues near the subunit interface (Figure 3). Aspartic acid was preferred at position 105 (D:N, 3:1), alanine at position 107 (A:Q, 4:1), and valine at position 109 (V:L, 3:1). All three of these residues are located along a single β strand directed toward the interface between dimers. These substitutions could improve streptavidin tetramerization, solubility, or thermodynamic stability. Further experiments to explore the functional contribution made by these substitutions should also consider the effects of the low

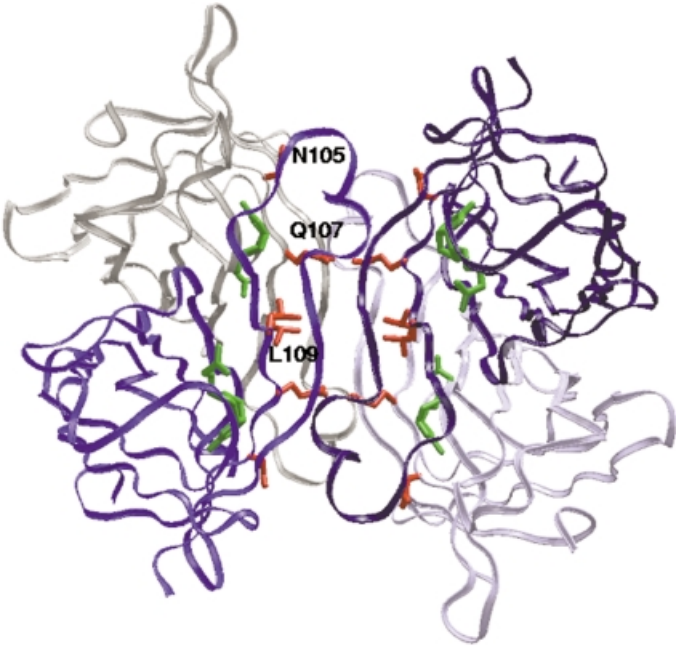

Figure 3. *Streptavidin tetramer with the side chains of the three residues for which substitution was preferred over the wild-type moeity highlighted. N105, Q107, and L109 were preferentially substituted with D, A, and V, respectively. For each mutation, four residues, one from each subunit, are shown in red. Green = biotin. PDB accession code 1SWE.*[29]

pH conditions used for elution of bound phage during selections. The side chains of these residues point toward the streptavidin β barrel exterior as opposed to lining the interior. These results suggest that residues in the interior of streptavidin are highly optimized, while surface residues are more amenable to variation. Such optimization of the interior residues could be required for core formation and long-range hydrophobic networking, as described above.

Data from shotgun scanning could thus be used to improve streptavidin for use in biotechnology. For example, the three residues with preference for non-wild-type substitutions could prove useful for optimization of recombinant streptavidin. The shotgun scanning data reported here also provide a map of residues important for streptavidin functionality. Residues identified by shotgun scanning could be the subject of further mutational studies to produce streptavidin with altered binding capabilities or other properties such as monomeric structure.

Conclusion

We report here for the first time systematic shotgun scanning of a receptor – small-molecule interaction. Shotgun scanning was used to determine the functional contribution of the 38 C-terminal residues of streptavidin to biotin binding. Shotgun scanning results were largely consistent with conventional site-directed mutagenesis studies for the few residues studied previously, which validates shotgun scanning as a high-throughput method for mapping receptor – small-molecule interactions. The results reported here also demonstrate the importance of previously unreported hydrophobic residues that contribute direct and indirect contacts with biotin. These include streptavidin residues likely responsible for formation of the β barrel and tetrameric structure of the protein. We expect these studies to inform protein structure prediction, de novo protein design, protein folding, and ligand design. Additional protein libraries of the remaining streptavidin residues could further contribute to our understanding of how every residue in a protein functions in molecular recognition.

Experimental Section

General: *E. coli* XL1-Blue and M13-VCS were obtained from Stratagene. *E. coli* SS320 is deposited with the American Type Culture Collection. Enzymes were from New England Biolabs, except for Taq polymerase, which was from Fisher. Succinimidyl – (biotinamido)-hexanoate-biotin (NHS-LC-biotin) was purchased from Pierce. BSA was from EM Science, Tween 20 was from Mallinckrodt, and *o*-phenylenediamine dihydrochloride was from Sigma. Anti-M13/horseradish peroxidase conjugate was obtained from Amersham Life Science. Deoxynucleotide triphosphates were obtained from ProMega, 10xPCR buffer was from Fisher, and Maxisorp microtiter plates were purchased from NUNC. Reagents for dideoxynucleotide sequencing were purchased from ABI/PE Biosciences.

Oligonucleotides: DNA degeneracies are represented by the IUB code (K = G/T, M = A/C, N = A/C/G/T, R = A/G, S = G/C, W = A/T, Y = C/T). The following oligonucleotides were used (mutation-encoding substitutions are shown in italics): sav-sg-1: 5′-CACGTGGAGCGGC-CAG*KMTGYTGGTGGTGCTGMAGCTSSTRYTRMCRCTSMAKSGSYTSYTRCT-KCCGGTRCTRCT*GAGGCCAACGCCTGGAAG-3′; sav-sg-2: 5′-CTGACCT-CCGGTACCACC*GMAGCTRMCGCTKSGRMAKCCRCTSYTGYTGGTSMTGM-TRCTKYTRCTRMAGYT*GTCTAGAGCGGTGGA-3′; SAV-F1: 5′-TGTAAAAC-GACGGCCAGTCGAGCACTTCACCAACAA-3′; SAV-R2: 5′-CAGGAAA-CAGCTATGACGACAACAACCATCGCCC-3′.

Construction of mutant SAV libraries: Phagemid pW1244c was used as the template for library construction. The phagemid is identical to a previously described phagemid[15] designed to display streptavidin on the surface of M13 bacteriophage as a fusion to a mutant P8 protein engineered for increased streptavidin display, with the following exception: codons encoding residues 115 and 116 of streptavidin were replaced by TAA stop codons. The phagemid was used as the template for oligonucleotide-directed mutagenesis as described by Kunkel[25] with the two oligonucleotides, sav-sg-1 and sav-sg-2, designed to simultaneously repair the stop codons and introduce mutations at the desired sites. Libraries 1 and 2 had theoretical diversities of 2.7×10^8 and 3.4×10^7 and experimental diversities of 2.5×10^8 and 6.8×10^8, respectively. Theoretical diversities were derived from the number of possible substitutions in each position of the library-encoding oligonucleotides and experimental diversities were obtained from phage library titers.

Screening for biotin binding activity: Biotinylated BSA was prepared by dissolving NHS-LC-biotin (1.7 mg, 3.7 μmol) in dimethyl sulfoxide (DMSO; 200 μL), quick transferal of the DMSO solution into BSA (5 mL, 5 mg mL^{-1}; 0.37 μmol), and incubation of the mixture at room temperature for 8 hours.

Phage from the streptavidin libraries were cycled through serial rounds of binding selection with biotinylated BSA and each library was subjected to separate rounds of selection. Maxisorp immunoplates (96-well) were coated with biotinylated BSA for one hour at

room temperature (100 μL, 10 μg mL^{-1} biotinylated BSA in 50 mM carbonate buffer (pH 9.6)). The plates were then blocked for 30 minutes with either BSA (0.2%) or casein (0.2%) in phosphate-buffered saline (PBS) and washed eight times with PT buffer (PBS, 0.05% Tween 20). The phage library in PBS (100 μL), BSA (0.1%), and Tween 20 (0.1%) was transferred to ten of the coated wells for the first round and two of the coated wells for successive rounds. After one hour, the plate was washed eight times with PT buffer. Phage were eluted by addition of HCl (100 μL, 100 mM) supplemented with streptavidin (0.05 mg mL^{-1}) and shaken vigorously for five minutes. The eluted phage were immediately neutralized with tris(hydroxymethyl)aminomethane – HCl (33 μL, 1.0 M; pH 8.0). Finally, remaining phage were eluted with XL1-Blue *E. coli* cells (200 μL). Half the eluted phage solution was used to infect XL1-Blue cells supplemented with carbenicillin (50 μg mL^{-1}) and M13-VCS helper phage (10^{10} phage mL^{-1}). The library selection was repeated three times.

Phage ELISA for determining biotin-binding clones: Phage ELISA protocols were adapted from a previous work.[26] Cultures of *E. coli* XL1-Blue harboring individual phagemids were grown in 96-well format for 18 hours at 37 °C in 2YT medium (1 mL), carbenicillin (50 μg mL^{-1}), and M13-VCS helper phage (10^{10} phage mL^{-1}). Cells were removed by centrifugation (10 min, 12 000 g) and the culture supernatant was used directly in the phage ELISA.

Maxisorp immunoplates (96-well) were coated with biotinylated BSA for one hour at room temperature as described above. The plates were then blocked for 30 minutes with BSA (0.2%) in PBS and washed eight times with PT buffer. Phage supernatant (100 μL) was then transferred to the coated wells. After two hours, plates were washed eight times with PT buffer, incubated with anti-M13/horseradish peroxidase conjugate (100 μL, 1:10 000) in PBS, BSA (0.1%), and Tween 20 (0.1%) for 30 minutes, and washed eight times with PT buffer and twice with PBS. Plates were developed by using a *o*-phenylenediamine dihydrochloride/H_2O_2 solution (100 μL, 1 mg mL^{-1}/0.02%), quenched with H_2SO_4 (50 μL, 2.5 M), and read spectrophotometrically at 492 nm.

DNA sequencing of positive clones: Culture supernatant that contained phage particles was used as the template for a PCR with oligonucleotides SAV-F1 and SAV-R2 as primers in order to amplify the streptavidin gene and incorporate universal sequencing primers. The amplified DNA fragment was used as the template in Big-Dye terminator sequencing reactions, which were analyzed on an ABI Prism 3700 capillary electrophoresis DNA sequencer. Data was tabulated by using the program SGCOUNT, as previously described.[13]

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