

Molecular Engineering of Streptavidin^a

TAKESHI SANO, SANDOR VAJDA, GABRIEL O. REZNIK,
CASSANDRA L. SMITH, AND CHARLES R. CANTOR

*Center for Advanced Biotechnology
and*

*Departments of Biomedical Engineering, Pharmacology, and Physics
Boston University
Boston, Massachusetts 02215*

INTRODUCTION

Streptavidin is a tetrameric protein produced by the bacterium *Streptomyces avidinii*; it has great similarity to the chicken protein avidin.¹⁻⁴ These proteins bind the vitamin biotin with an extremely high affinity. The dissociation constant of streptavidin-biotin and avidin-biotin complexes is estimated at around 10^{-15} M;^{3,4} this is one of the tightest noncovalent interactions found in biological systems. Streptavidin and avidin are among the most stable proteins known; these proteins are resistant to, for example, high temperatures, extremes of pH, organic denaturants, and proteolytic enzymes. These characteristics generate considerable protein chemical interest, making streptavidin and avidin unique models in studying high-affinity macromolecule-ligand interactions and the structural stability of proteins in general. For example, one may ask why the interaction between these proteins and biotin is so tight and why these proteins are structurally so stable. The tetrameric nature of these proteins provides additional structural interest.

We have been doing rational structural and functional studies to answer such questions about streptavidin. Streptavidin has neither cysteine residue nor carbohydrate moieties, whereas avidin has one disulfide bridge and one *N*-linked oligosaccharide chain per subunit.^{3,4} These differences make streptavidin a more easily manipulated target using recombinant DNA technology, thereby facilitating systematic studies.

STRUCTURES OF STREPTAVIDIN AND ITS BIOTIN-BINDING SITE

The three-dimensional structure of streptavidin, obtained by X-ray crystallography^{5,6} (FIGURE 1), has helped enormously to understand the structural characteristics of this protein. Each streptavidin subunit consists of eight β -strands, forming a very stable β -barrel structure with a simple antiparallel topology. Four subunits are positioned in dihedral D_2 symmetry to form a tetramer, which can be viewed as a dimer of a stable subunit dimer. A pair of subunits is associated very tightly to form a stable dimer, in which the subunit barrel surfaces have complementary curvatures,

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making numerous intersubunit van der Waals interactions. A tetramer is formed by two stable dimers that are associated relatively weakly via a small intersubunit contact area that contains hydrogen bonds and van der Waals interactions. Thus, each streptavidin molecule has two different subunit interfaces; one is the strong interface between subunits in a stable dimer and the other is the weak interface between two stable dimers (dimer-dimer interface). The biotin-binding site is located at one end of each β -barrel near the dimer-dimer interface.

A number of amino acid residues make contact with biotin, providing the extremely high stability of the streptavidin-biotin complex. The biotin-binding pocket is exquisitely positioned to provide a precise fit to biotin both geometrically and electrostatically. The amino acid residues that make hydrogen bonds or strong van der Waals contacts with biotin are shown schematically in FIGURE 2, and the interaction energies between these residues and biotin are listed in TABLE 1. Eight amino acid residues make hydrogen bonds with different parts of the biotin. The residues that form hydrogen bonds are Asn-23, Ser-27, and Tyr-43 with the ureido oxygen of biotin; Ser-45 and Asp-128 with each of the ureido amino groups; Thr-90 with the thiophene sulfur; and Asn-49 and Ser-88 with the carboxyl oxygens. Four tryptophan residues, Trp-79, -92, -108, and -120, together with Leu-25, Val-47, and Leu-110, form hydrophobic parts of the biotin-binding pocket, which interact with the thiophene ring and the alkyl chain of biotin through van der Waals forces. Interestingly, one of these residues, Trp-120, is not derived from the subunit binding to biotin, but instead is from an adjacent subunit through the dimer-dimer interface.

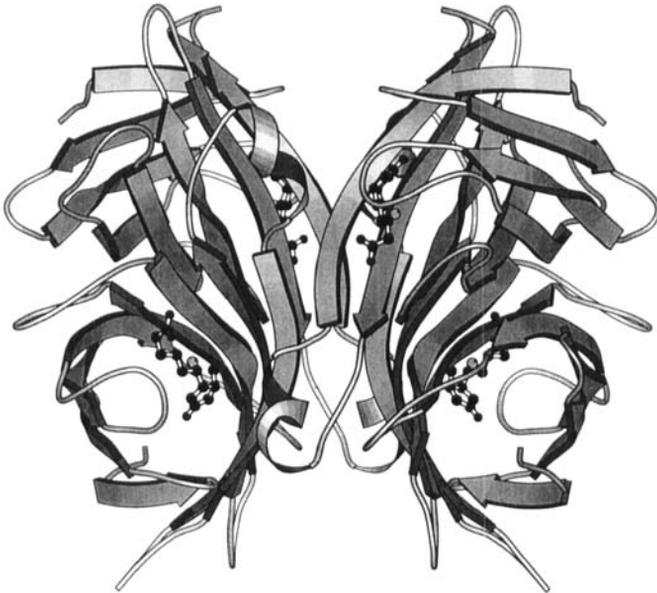


FIGURE 1. Schematic illustration of tetrameric streptavidin with four bound biotins. This picture is drawn based on the known three-dimensional structure of the natural core streptavidin-selenobiotin complex, obtained by X-ray crystallography.⁶

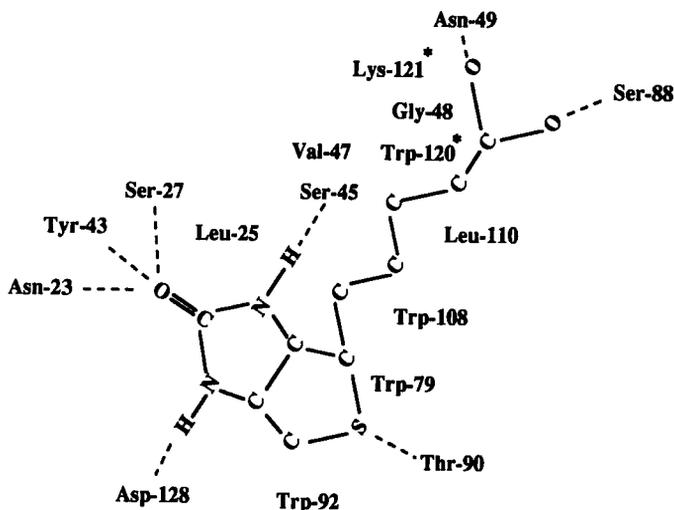


FIGURE 2. Schematic illustration of biotin and the biotin-binding site of streptavidin. Hydrogen bonds between biotin and amino acid residues of streptavidin are shown by dashed lines. Trp-120 and Lys-121, marked with asterisks, are derived from an adjacent subunit through the dimer-dimer interface. Modified from reference 7 with permission.

Similarly, Lys-121, which makes electrostatic interaction with the carboxyl group of biotin, is also provided by an adjacent subunit. These intersubunit contacts play key roles in the extremely tight biotin binding by this tetrameric protein. Another interesting structural feature is that a loop consisting of the sequence from Ser-45 to Ala-50 is likely to be relatively flexible,⁵ but it is ordered upon biotin binding, whereupon Val-47, Gly-48, and Asn-49 on the loop make strong electrostatic and van der Waals interactions with biotin.

CLONING AND EXPRESSION OF THE STREPTAVIDIN GENE

Several years ago, we cloned the gene for streptavidin in *Escherichia coli*.⁸ Then, attempts were made to express the cloned streptavidin gene by using bacterial expression systems. Active streptavidin is extremely toxic to any cell in which it is expressed because it tightly binds cellular biotin, which is essential for cell growth and viability. Thus, many expression systems showed very low expression efficiencies or, in some cases, host cells could not even maintain expression plasmids carrying the streptavidin gene stably. However, by using the bacteriophage T7 expression system, which has very tight control in expressing target genes,⁹ a cloned streptavidin gene was expressed very efficiently in *E. coli*, and expressed streptavidin accounted for greater than 25% of the total cell protein.¹⁰

Expressed streptavidin formed inclusion bodies, as seen in many bacterial overexpression systems. However, solubilization of the inclusion body fraction, followed by renaturation, generated tetrameric streptavidin with full biotin-binding

ability.¹⁰ The establishment of efficient expression and purification systems for streptavidin allowed us to modify, systematically, the structure of streptavidin by genetic engineering to fully understand the structural and functional characteristics of this protein.

DESIGN OF STREPTAVIDIN MUTANTS

The known three-dimensional structure of streptavidin^{5,6} is of great assistance in designing streptavidin mutants. In addition, the use of computational molecular modeling methods, based on the known three-dimensional structure, allows rational

TABLE 1. Electrostatic, van der Waals, and Total Interaction Energies between Biotin and Amino Acid Residues of Streptavidin^a

Residue	Interaction Energy (kcal/mol)		
	Electrostatic	van der Waals	Total
Asn-23	-1.14	-0.92	-2.07
Leu-25	-0.10	-1.60	-1.70
Ser-27	-2.19	-0.55	-2.74
Ser-45	-0.94	-2.17	-3.11
Val-47	0.12	-2.52	-2.40
Gly-48	-0.91	-1.54	-2.46
Asn-49	-1.78	-2.23	-4.01
Trp-79	0.05	-4.51	-4.46
Ser-88	-2.68	-0.84	-3.52
Thr-90	-0.00	-1.23	-1.23
Trp-92	0.17	-1.69	-1.52
Trp-108	-0.03	-2.34	-2.37
Leu-110	-0.02	-2.42	-2.45
Trp-120 ^b	-0.01	-1.61	-1.62
Lys-121 ^b	-1.20	-0.27	-1.47
Asp-128	-1.26	-1.14	-2.40

^aThe electrostatic and van der Waals interaction energies between biotin and each amino acid residue were calculated using the X-ray structure of the streptavidin-selenobiotin complex⁶ and the CHARMM molecular mechanics program with the CHARMM-19 parameter set. A distance-dependent dielectric of $4r$ was used in the calculation. Nonpolar hydrogen atoms were added to the structure using the HBUILD option of CHARMM. The resulting streptavidin tetramer with four bound biotins was subjected to 200 steps of Newton-Raphson minimization in order to eliminate possible steric overlaps.

^bTrp-120 and Lys-121 are derived from an adjacent subunit through the dimer-dimer interface.

design of streptavidin mutants. In almost all cases, streptavidin mutants, which were predicted to be stable and active after the change of one or more amino acid residues, behaved as predicted. Here, we describe a few streptavidin mutants, which were recently designed, and the structural and thermodynamic considerations behind the design of these mutants.

A Minimum-sized Core Streptavidin

When streptavidin is produced by *S. avidinii*, it is secreted as a precursor and then its signal peptide is removed. The resulting mature protein is extremely susceptible to proteolysis and thus both the N- and C-termini are readily truncated to yield so-called core streptavidin.^{11,12} However, natural core streptavidin preparations are often heterogeneous and such structural heterogeneity can be observed even within single tetrameric molecules. Thus, it would be desirable to make structurally homogeneous streptavidin for systematic studies on this protein. In particular, we were interested in designing a minimum-sized core streptavidin that might have enhanced properties because of the removal of any nonfunctional terminal sequences that are located on the surface of the molecule.

Most natural core streptavidin molecules consist of the sequences from around Ala-13 to around Ser-139, but the identity of the terminal residues varies. The sequences of natural core streptavidins are not necessarily those that are structurally most favorable; instead, they may merely indicate that the sequences around amino acids Ala-13 and Ser-139 are particularly susceptible to proteolysis. In fact, the X-ray crystallographic studies on natural core streptavidin were able to refine the structure only from Ala-13 or Glu-14 to Val-133 without the terminal regions,^{5,6} which corresponds almost perfectly to the stable β -barrel structure consisting of the sequence from Gly-19 to Val-133. This indicates that the terminal regions, present in core streptavidin, are rather disordered,⁵ which suggests that they have little contribution to the fundamental properties of streptavidin, such as biotin binding, formation of the β -barrel structure, and subunit association. This prediction encouraged us to remove all of the terminal sequences, which have no apparent function, to design a minimum-sized core streptavidin. The recombinant core streptavidin that we designed has the amino acid sequence from Gly-16 to Val-133¹³ and thus consists of almost only the β -barrel structure characteristic of streptavidin.

This minimum-sized core streptavidin is tetrameric and binds one biotin per subunit, as does natural streptavidin. The truncation of the terminal residues had no appreciable effects on the solubility characteristics of the protein, unlike those present in mature, full-length streptavidin, which cause the formation of higher-order aggregates. However, this minimum-sized core streptavidin showed enhanced stability against denaturation by strong organic denaturants. For example, this molecule retained greater than 80% of its biotin-binding ability in the presence of 6 M guanidine hydrochloride at pH 1.5, under which conditions natural core streptavidin lost almost all of its biotin-binding ability. In addition, this core streptavidin had higher biotinylated DNA-binding ability than natural core streptavidin. These results suggest that the terminal regions, present on the surface of natural core streptavidin, reduce the overall stability of streptavidin and prevent biotinylated macromolecules from approaching the biotin-binding sites.

A Streptavidin Mutant with a Reduced Biotin-binding Affinity

Trp-120 makes intersubunit hydrophobic contacts to biotin bound by an adjacent subunit through the dimer-dimer interface. This intersubunit contact is likely to make a key contribution to the high biotin-binding affinity of streptavidin because

dissociated subunits have much lower affinity for biotin as seen by electrophoretic analysis of natural streptavidin¹⁴ and because immobilized monomeric avidin is known to release biotin under relatively mild conditions.¹⁵ Interaction energy calculations (TABLE 1) also show that this residue makes relatively strong van der Waals interactions with biotin. To understand the role of the intersubunit contacts made by Trp-120 to biotin in the properties of streptavidin, we designed a streptavidin mutant, in which Trp-120 is replaced with phenylalanine.¹⁶

This W120F streptavidin mutant can still bind biotin tightly enough to fully retain it even in the presence of high concentrations of urea at neutral pH or in a wide pH range. However, its biotin-binding ability was reduced to the point where bound and free biotins show facile exchange under physiological conditions. The biotin-binding affinity of this mutant was estimated to be $1-3 \times 10^8 \text{ M}^{-1}$, indicating the significant contribution of the intersubunit contacts made by Trp-120 to biotin binding.

Biotin binding by natural streptavidin strengthens the dimer-dimer association by approximately 5 kcal/mol. Electrophoretic analysis of the W120F mutant in the presence of sodium dodecyl sulfate indicates that its subunit association is not tightened significantly upon biotin binding, unlike natural streptavidin. The reduced effect of biotin binding on the subunit association of this mutant is apparently caused by the lack of the intersubunit contacts made by Trp-120 to biotin through the weak dimer-dimer interface.

Cross-linked Streptavidins with Tighter Subunit Association

Tetrameric streptavidin has two subunit interfaces—the strong interface between subunits in a stable dimer and the weak dimer-dimer interface. If the subunit association, particularly at the weak dimer-dimer interface, could be tightened, the resulting streptavidin might retain bound biotin more stably even under relatively harsh conditions because the maintenance of the tetrameric structure, particularly the dimer-dimer interface, is essential for the intersubunit contacts made by Trp-120 and Lys-121 to biotin. To enhance the subunit association of streptavidin, specific covalent cross-links were introduced into pairs of subunits through the dimer-dimer interface.¹⁷

In natural streptavidin, His-127 residues from two adjacent subunits are in close proximity across the weak dimer-dimer interface of the tetramer.^{5,6} Replacing His-127 with cysteine (H127C) generated a fully active, tetrameric protein that can spontaneously form two disulfide bonds through the weak dimer-dimer interface under mild oxidizing conditions. The two pairs of adjacent cysteine residues can also be cross-linked irreversibly by a sulfhydryl-specific bifunctional cross-linker. The successful cross-linking of Cys-127 residues through the dimer-dimer interface without disturbing the biotin-binding ability indicates that two Cys-127 residues are located in close proximity, suggesting that no significant changes in local structure occurred by the H127C mutation or by cross-linking between Cys-127 residues.

Another cross-linked mutant was made by first assembling a heterotetramer consisting of two species of subunits in a 1:1 ratio; one has lysine at position 127 and the other has aspartic acid. These tetramers apparently formed predominantly with Lys-127 and Asp-127 apposed across the weak dimer-dimer interface; this configuration should be electrostatically more favorable than those having the same amino

acid residues facing each other at the interface. This proposal has been confirmed by successful, almost quantitative, cross-linking of the amino group of Lys-127 and the carboxyl group of Asp-127 by a zero-length cross-linker, a water-soluble carbodiimide. The resulting cross-linked molecule was essentially fully active.

These cross-linked streptavidins showed enhanced resistance to various denaturing conditions. For example, these streptavidins remained mostly tetrameric after heat treatment at 80 °C in the absence of biotin, under which conditions natural core streptavidin dissociated completely into subunits. The cross-linked molecules retained bound biotin more stably than their counterparts without cross-links or natural core streptavidin in the presence of high concentrations of guanidine hydrochloride at very acidic pH. These results demonstrate that the introduction of covalent bonds between adjacent subunits through the weak dimer-dimer interface enhances the overall stability of streptavidin. They also reveal the importance of the intersubunit contacts made by Trp-120 and Lys-121 to biotin through the dimer-dimer interface in biotin binding by streptavidin, which is consistent with the results seen with the W120F mutant with a reduced biotin-binding affinity.

PERSPECTIVES

Several streptavidin mutants have been described that were designed to understand the structural and functional characteristics of streptavidin. Because of the unusual properties of streptavidin, such as the extremely tight ligand-binding ability and very high structural stability, further, more extensive studies on this protein should not only provide deeper understanding of this attractive protein, but should also offer useful insights into the structural characteristics of high-affinity macromolecule-ligand interactions and the stability of proteins in general. In addition, many of the streptavidin variants produced will also be very useful as biological tools in biotechnological applications of the streptavidin-biotin system.

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