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Review

Genetic engineering of streptavidin, a versatile affinity tag

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Abstract

Streptavidin, a tetrameric protein produced by *Streptomyces avidinii*, has been used as a useful, versatile affinity tag in a variety of biological applications. The efficacy of streptavidin is derived from its extremely high binding affinity for the vitamin biotin. For the last several years, we have used genetic engineering as a primary means to enhance the properties of streptavidin and to expand the application of streptavidin as an affinity tag. In this review, we describe several genetically engineered streptavidin variants, which include a streptavidin with a reduced biotin-binding affinity, a dimeric streptavidin, and a fusion protein between streptavidin and protein A, along with their potential applications in biological science.

Keywords: Reviews; Genetic engineering; Streptavidin; Biotin; Fusion proteins

Contents

1. Introduction

Streptavidin is a tetrameric protein produced by *Streptomyces avidinii* [1,2]. It binds up to four molecules of a small water-soluble vitamin, D-biotin (vitamin H), with a remarkably high affinity [3,4]. The dissociation constant (K_d) of the complex between streptavidin and biotin is estimated at approximately 10^{-15} *M*, which is one of the tightest noncovalent interactions known between proteins and their ligands. Streptavidin is also one of the most stable proteins known. For example, it can maintain its functional structure at high temperatures, extremes of pH, and in the presence of high concentrations of denaturants and organic solvents. This protein also has exceptional stability against proteolysis. These unique properties of streptavidin,

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along with the ability of biotin to be incorporated easily into various biological materials, allow streptavidin to serve as a versatile, powerful affinity tag in a variety of biological applications [5,6]. The versatility of the applications of streptavidin and its related protein, avidin, has clearly been documented by a literature survey [6]. In particular, streptavidin is one of the most frequently used proteins in clinical diagnostics.

For the last several years, we have designed and produced a wide variety of streptavidin variants by using genetic engineering [7–10]. These streptavidin variants have enhanced properties over the natural protein, and thus they have the potential to serve as more useful, versatile affinity tags in biological applications. In this article, we describe several genetically engineered streptavidin variants and their potential applications.

2. Genetic engineering of streptavidin

About a decade ago, we cloned the gene for streptavidin in *Escherichia coli* from a genomic library of *S. avidinii* [11]. Then, attempts were made to express the cloned streptavidin gene by using bacterial expression systems. The extremely tight binding affinity for biotin, which is essential for cell growth and viability, makes streptavidin extremely toxic to any cell in which it is expressed. Thus, in many cases, host cells were unable to maintain expression plasmids carrying the streptavidin gene stably. This suggested that very tight expression control is essential for this toxic gene to be expressed efficiently. However, an enhanced version of the T7 expression system [12,13], in which constitutive expression of T7 lysozyme, a natural inhibitor of T7 RNA polymerase [14], is used to repress the expression of the target gene under the control of a T7 promoter, allows streptavidin to be produced very efficiently in *E. coli*; expressed streptavidin accounts for greater than 30% of total cell protein [15]. Expressed streptavidin generally forms inclusion bodies, as seen with many *E. coli* over-expression systems, but it is easily solubilized by treatment with an organic denaturant, such as guanidine hydrochloride. Solubilized streptavidin is renatured by slow removal of the organic denaturant and then purified by affinity chromatography using 2-iminobiotin [16], a biotin analog which binds to streptavidin reversibly in a pH-dependent manner under relatively mild conditions. The resulting streptavidin is tetrameric and binds one biotin per subunit as does natural streptavidin, indicating that these expression and purification methods can produce active streptavidin. At least a few milligrams of active, purified streptavidin can be obtained from only 100 ml of culture by using these methods. Establishment of efficient expression and purification methods for recombinant streptavidin has allowed the design and production of a variety of streptavidin variants by using genetic engineering.

In attempting to enhance the properties of streptavidin, we have taken two basic strategies. One strategy is to enhance the properties of streptavidin itself by genetic engineering. The other is to make recombinant fusion proteins between streptavidin and partner proteins, in which the fused partner proteins should acquire highly specific, strong biological recognition capability derived from the streptavidin moiety. Obviously, the first and second strategies can be combined to make streptavidin-containing fusion proteins, which carry genetically engineered versions of streptavidin.

In designing streptavidin variants, computational molecular simulation and modelling methods, including binding free energy calculations [17–19], have been used successfully. These computational methods, based on the known three-dimensional structure of streptavidin [20,21] (Fig. 1), allow rational design of a variety of streptavidin variants. This greatly facilitates the entire effort by significantly reducing the number of variants which need to be actually produced and experimentally tested to achieve the desired properties. In almost all cases, streptavidin variants that were predicted to be stable and active by these methods after the change of up to a few amino acid residues behaved as predicted.

3. A streptavidin with a reduced biotin-binding affinity

One distinct characteristic of streptavidin is its extremely high binding affinity for biotin. This

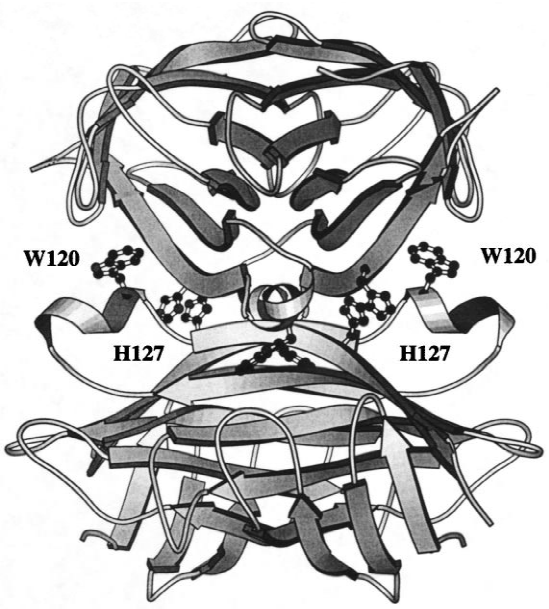

Fig. 1. Backbone structure of a tetrameric streptavidin, based on the crystal structure of streptavidin. The side chains of W120 and H127 residues are also shown. This is drawn based on the X-ray structure of natural streptavidin [20,21] by using MOLSCRIPT [47]. Reproduced from [31] with permission [copyright (1997) National Academy of Sciences, USA].

allows biotinylated targets to be captured very tightly and specifically by streptavidin or its immobilized form. However, the almost irreversible biotin binding by streptavidin makes release of captured biotinylated targets virtually impossible without the use of extremely harsh conditions, such as in the presence of 6 *M* guanidine hydrochloride at pH=1.5, under which streptavidin is completely denatured. This makes streptavidin unusable for purification and isolation of unstable biotinylated targets, in which the captured targets must be released in functional form maintaining their structures and activities. For example, a biotinylated growth factor could be used to isolate its receptor [22], for which the biotinylated growth factor–receptor complex is captured by a streptavidin-coated solid surface, such as microtiter plates, gel matrices, and magnetic microbeads. However, the need for harsh conditions to release the biotinylated growth factor from the streptavidin-coated solid surface makes the isolation of the growth factor–receptor complex in functional form essentially impossible. It would be desirable if captured biotinylated targets could be released from streptavidin under mild conditions which allow the maintenance of the functions and structures of the targets. To achieve this goal, we attempted to design a streptavidin variant with the biotin-binding affinity high enough to bind to biotinylated targets tightly but reduced to the level where bound biotin can be exchanged efficiently with free biotin.

In natural streptavidin, each W120 residue makes hydrophobic contacts to biotin bound by an adjacent subunit through the dimer–dimer interface (Fig. 1) [20,21]. This inter-subunit contact made by W120 has a significant contribution (approximately −1.6 kcal/mol) [9] to the extremely tight biotin binding by streptavidin. Replacing W120 with F120 could reduce these inter-subunit contacts to biotin considerably because of the substantially reduced hydrophobicity of F120, while maintaining the hydrophobic environment of the biotin-binding site [23]. Binding free energy calculations predicted that this W120F mutation could reduce the inter-subunit contacts to biotin to the point where bound and free biotins could show facile exchange at neutral pH.

A streptavidin variant with the W120F mutation still binds to biotin tightly [23]. For example, bound biotin can be retained by this variant in a pH range from 3 to 11 or in the presence of urea at up to 6 *M* at neutral pH. However, the binding is reduced to the point where bound biotin can be exchanged efficiently with free biotin. For example, greater than 50% of bound biotin was released in 20 min at 21°C from this streptavidin variant by the addition of free biotin at 330 μ*M* at pH=7.4. By equilibrium dialysis analysis, the biotin-binding affinity (K_d) of this variant was estimated at about 10^{-8} *M*, which is equivalent to the antigen-binding affinities of many antibodies. This reveals that the biotin-binding affinity was reduced by several orders of magnitude by the W120F mutation. The reduced biotin-binding affinity of this streptavidin variant should be enormously useful in purification and isolation of unstable biotinylated targets. For example, immobilized form of this streptavidin variant can capture biotinylated targets tightly, and the captured targets can easily be released from immobilized streptavidin by simply adding excess biotin under physiological conditions.

4. A dimeric streptavidin

When streptavidin is used in vivo, such as for tumor imaging [24–29], it shows some undesirable pharmacokinetics. For example, streptavidin clears from the circulation slowly, and it accumulates non-specifically in the kidney and liver [30]. This unfavorable in vivo behavior of streptavidin is attributable, at least partly, to its large size (approximately 54 kDa per tetramer). If the size of streptavidin could be reduced considerably without disturbing its properties, streptavidin could become a more effective in vivo targeting reagent in medical applications. In one attempt to reduce the size of streptavidin, we designed a dimeric streptavidin [31].

The design of a dimeric streptavidin was initiated by a single amino acid mutation. In natural streptavidin, the side chains of a pair of H127 residues are in close proximity (approximately 3 Å) at the dimer–dimer interface [20,21], at which a pair of stable dimers are associated to form a tetramer (Fig. 1). Introduction of a charged amino acid at the position of H127 ought to prevent a pair of stable dimers from forming a tetramer due to electrostatic repulsion between the charged amino acid residues. Binding free energy calculations indicated that the electrostatic interaction energy between a pair of stable dimers should be increased by the H127D mutation from −5.8 kcal/mol to +14.6 kcal/mol, sufficient to prevent the association of two stable dimers. However, this streptavidin derivative formed insoluble aggregates in aqueous media. This was very likely caused by the exposure of the hydrophobic dimer–dimer interface, which is buried in tetrameric streptavidin but would be exposed to solvent in a dimeric streptavidin. In agreement with the experimental results, the desolvation contribution to the binding free energy between a pair of stable dimers was almost unaffected by the H127D mutation (from −68.9 kcal/mol to −64.2 kcal/mol), and it is too high for this variant to be soluble in aqueous media.

To increase the solubility of this insoluble derivative in aqueous media by reducing the hydrophobicity of the dimer–dimer interface, an entire loop region from G113 to W120 was truncated. This hydrophobic loop should be exposed to solvent and have no apparent contact to biotin in a dimeric streptavidin. Placing a β-turn between the two β-strands, connected by this loop in tetrameric streptavidin, should have minimal effects on the structure of streptavidin. Binding free energy calculations showed that the loop truncation should increase the desolvation contribution to the binding free energy between a pair of stable dimers from −68.9 kcal/mol to −23.4 kcal/mol, while the positive electrostatic interaction energy, essential for dimer formation, is maintained (+8.4 kcal/mol).

The streptavidin variant containing the G113–W120 loop deletion, in addition to the H127D mutation, formed a soluble dimeric streptavidin. This demonstrates that the loop deletion has successfully improved the solubility characteristics. The biotin-binding affinity (K_d) of this dimeric streptavidin was estimated at approximately 10^{-7} *M* at 4°C at pH= 7.4; this reduced biotin-binding affinity is caused by the lack of the inter-subunit contacts made by W120 and K121 to biotin through the dimer–dimer interface in tetrameric streptavidin. This dimeric streptavidin was stable in the presence of biotin. However, prolonged storage of this streptavidin variant without biotin caused the dissociation of dimers into monomers, followed by the formation of insoluble aggregates. This suggests that the deletion of the G113–W120 loop may have destabilized the subunit association of the dimer. Binding free energy calculations showed that the electrostatic interaction between subunits in the dimeric streptavidin is weakened by the G113–W120 loop deletion (from −21.8 kcal/mol without the loop deletion to −12.8 kcal/mol), and this is why this dimeric streptavidin is unstable in the absence of biotin. Additional mutations are now being introduced into this dimeric streptavidin to restore the tight subunit association.

The biodistribution of this dimeric streptavidin was tested to see if it could show better in vivo behavior than tetrameric streptavidin. The dimeric streptavidin (23.6 kDa per molecule), along with a minimum-sized tetrameric core streptavidin (50.4 kDa per molecule) [32], was labeled with ^{35}S through its primary amino groups and administered intravenously into normal CD-1 male mice through tail vein, followed by quantitation of radioactivity in tissues and blood by liquid scintillation counting. Preliminary studies showed that the dimeric streptavidin had much faster clearance from the circula-

tion and lower accumulation in the liver and kidney than the minimum-sized tetrameric core streptavidin. For example, the radioactivity remaining in the circulation at 3 h after administration was reduced from 0.7% of the injected dose per gram tissue for the tetrameric streptavidin to only about 0.1% for the dimeric streptavidin. Similarly, accumulation in the liver was reduced from 0.3% to less than 0.1%. This suggest that this dimeric streptavidin could serve as a more effective in vivo targeting reagent than tetrameric streptavidin, although other potential problems, such as the presence of endogenous biotin [33] and the immunogenicity of streptavidin [34] when it is used repeatedly, along with the stability and biotin-binding affinity of this dimeric streptavidin, will also need to be solved.

The C_2 symmetry of this dimeric streptavidin should also be useful when it is fused to some partner proteins. For example, when streptavidin is fused to a trans-membrane protein, the D_2 symmetry of tetrameric streptavidin allows only two trans-membrane protein moieties of the resulting fusion to span the cell membrane, leaving two remaining moieties exposed to the extracellular or intracellular space. In contrast, the C_2 symmetry of the dimeric streptavidin should allow the two trans-membrane protein moieties of the fusion to span the cell membrane in a manner similar to the parental protein with the streptavidin moiety located at the extracellular (or intracellular) space.

5. A streptavidin-protein A fusion protein

A recombinant fusion protein between streptavidin and a partner protein, if successfully produced, should be very useful because it has two independent functionalities, one domain derived from its streptavidin moiety and the other from the partner protein moiety. Such a recombinant fusion protein should be structurally homogeneous and easily produced in large quantities, as opposed to chemical conjugates between streptavidin and the partner protein which often display structural heterogeneity and are difficult to produce in large quantities.

One of the partners we used for streptavidin-containing fusion proteins is protein A produced by *Staphylococcus aureus*. Protein A binds to the Fc domain (constant region) of an immunoglobulin G (IgG) without disturbing its antigen-binding ability [35–37]. Thus, protein A is widely used in immunological applications, such as purification of antibodies, host- and subclass-specific detection of antibodies, and detection of biological materials through their antibodies. Fusion of a portion of the protein A gene to the streptavidin gene allowed the production of a fusion protein which binds both biotin and IgG independently [38]. This fusion protein is tetrameric, and it binds four biotins and four IgG's per molecule. The bispecific binding abilities for biotin and IgG allow the preparation of specific antibody conjugates by simply mixing antibodies and biotinylated molecules with the fusion protein at appropriate ratios without the need for covalent chemistry. It could also offer various other applications, such as immobilization of antibodies or antibody–antigen complexes on solid surfaces, production of multivalent antibodies still capable of binding to biotinylated molecules, and production of bispecific multivalent antibodies.

One application of this fusion protein in which we were particularly interested is the production of specific antibody–nucleic acid conjugates. Both antibodies and nucleic acids have highly specific binding abilities for their antigens and complementary sequences, respectively, and the binding abilities of antibodies and nucleic acids are used independently in various biological assays [39]. Combining these two independent binding abilities could allow the development of an assay system with the performance greater than those obtainable by using each of these binding abilities individually. This basic idea allowed us to develop the concept of immuno-PCR (polymerase chain reaction) [40–46]. Immuno-PCR is an antigen detection system, in which a purely arbitrary marker DNA is targeted to antigen–antibody complexes. Segments of the marker DNA can be amplified specifically by PCR or other in vitro nucleic acid amplification techniques, resulting in the production of large amounts of specific nucleic acid fragments that can be detected by various sensitive detection methods available for nucleic acids. The key component in this scheme is the linker that allows specific targeting of marker DNA to antigen–antibody complexes. The fusion protein between

streptavidin and protein A, described above, should be ideal to serve as the linker; its bispecific, independent binding abilities for biotin and IgG should allow specific targeting of any biotinylated DNA to antigen–antibody complexes.

This immuno-PCR scheme was tested by using a procedure very similar to conventional enzyme-linked immunosorbent assays (ELISA). A conjugate between the streptavidin-protein A fusion protein and a biotinylated marker DNA, prepared by simply mixing the two components at an appropriate ratio, was used as a counterpart of an enzyme-conjugated secondary antibody in ELISA. A segment of the marker DNA, attached to the antigen through the antibody and the fusion protein, was amplified by PCR which serves as the signal amplification system in immuno-PCR, equivalent to enzyme reactions in ELISA. The enormous amplification capability of PCR, along with the use of sensitive detection systems for nucleic acids, allows considerable enhancement in sensitivity of antigen detection. In a model system, in which agarose gel electrophoresis was used for detection of PCR products, only 10^{-21} to 10^{-22} mol (fewer than 10^3 molecules) of antigens were specifically and reproducibly detected. This sensitivity is several orders of magnitude greater than that of standard ELISA. Further enhancement in detection sensitivity should be achievable by, for example, using greater numbers of PCR amplification cycles and more sensitive detection methods for PCR products. These suggest that immuno-PCR offers the great potential to become a standard method for ultra-sensitive detection of targets in biological assays. In particular, one of the most practical applications of immuno-PCR is to clinical diagnostics. Its extremely high sensitivity should enable the specific detection of rare antigens that are present only in very small numbers; this should allow, for example, the diagnosis of infections and diseases at earlier stages of infection and disease development than possible with other methods. Fully automated immuno-PCR systems could also be developed because of the simplicity of immuno-PCR, and such automated systems, if successfully developed, will be enormously useful in clinical diagnostics, in which large numbers of samples need to be analyzed repeatedly.

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