Streptavidins with intersubunit crosslinks have enhanced stability

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Received 11 January 1996; accepted 8 May 1996.

Natural tetrameric streptavidin has two subunit interfaces; one is a strong interface between subunits in a tightly associated dimer, and the other is a weak interface between a pair of such dimers (dimer-dimer interface). To test whether strengthening the weak dimer-dimer interface could provide streptavidin with additional structural stability, covalent crosslinks were introduced between adjacent subunits through the dimer-dimer interface. Specific crosslinking sites were designed by site-directed mutations of His-127 residues that are in close proximity in natural streptavidin. The first and second streptavidin constructs have a disulfide bond and an irreversible covalent bond, respectively, between two Cys-127 residues across the dimer-dimer interface. The third variant is a hybrid tetramer consisting of two different streptavidin species, one having lysine and the other aspartic acid at position 127, which are covalently crosslinked. All streptavidin constructs with intersubunit crosslinks showed higher biotin-binding ability than natural core streptavidin after heat treatment. All of these crosslinked streptavidins retained bound biotin more stably than natural core streptavidin in guanidine hydrochloride at very acidic pH. These results suggest that the introduction of covalent bonds across the dimer-dimer interface enhances the overall stability of streptavidin.

Keywords: streptavidin, protein stability, chemical crosslinking, protein engineering

Streptavidin, a tetrameric protein isolated from *Streptomyces avidinii*, binds D-biotin with an exceptionally high affinity ($K_a \sim 10^{15}$ M^{-1})[1,2]. The known three-dimensional structure of this protein[3,4] shows that a pair of subunits is associated very tightly to form a stable dimer, in which the subunit β-barrel surfaces have complementary curvatures making numerous intersubunit van der Waals interactions. A tetramer is formed by two stable dimers that are associated relatively weakly by van der Waals and electrostatics interactions across a small intersubunit contact area. Of the two subunit interfaces in streptavidin, one interface is between subunits in a stable dimer, and the other is between two stable dimers (dimer-dimer interface). Interaction energy calculations on the subunit association of streptavidin indicate that the stability of the stable dimer subunit interface is significantly greater than that of the dimer-dimer interface (S. Vajda, unpublished data). When the dissociation of streptavidin occurs, it is likely that the dimer-dimer interface would be disrupted first because of its lower stability. If such dissociation occurred, it is likely that the resulting dimeric molecules would have much lower affinity for biotin because of the lack of contacts made by Trp-120 of an adjacent subunit with biotin through the dimer-dimer interface[5,6]. If the subunit association of streptavidin could be tightened, particularly at the dimer-dimer interface, the resulting molecule would likely retain bound biotin more stably under conditions that induce subunit dissociation.

To stabilize the subunit association of streptavidin, we have introduced covalent bonds between adjacent subunits through the dimer-dimer interface. These covalent bonds will prevent the dissociation of the tetramers along the dimer-dimer interface; this might allow streptavidin to retain bound biotin even under relatively harsh conditions. Streptavidin constructs containing intersubunit crosslinks were characterized to see the effect of the intersubunit covalent bonds on the overall stability of streptavidin.

Results and discussion

Design of streptavidins with intersubunit crosslinks. To enhance the subunit association of streptavidin, covalent bonds were introduced between adjacent subunits through the dimer-dimer interface. The known three-dimensional structure of the protein[3,4] shows that His-127 of one subunit faces the same amino acid of the adjacent subunit across the dimer-dimer interface. This suggests that amino acid substitutions at position 127 could be used to introduce a covalent bond between adjacent subunits. The resulting molecules should have two covalent bonds between two pairs of subunits. Because of the covalent bonds between subunits, the dissociation of the molecule along the weak dimer-dimer interface could be prevented; this might provide streptavidin with enhanced structural stability.

Three independent amino acid substitutions were carried out by replacing His-127 with cysteine, lysine, or aspartic acid. Stv-C127, which contains a unique cysteine residue at position 127, was designed to generate two species of crosslinked streptavidins. One construct has a disulfide bond between adjacent subunits through the dimer-dimer interface, and the other contains an irreversible covalent bond between the two sulfhydryl groups. A similar construct with a disulfide bond between Cys-127 residues was recently designed by Chilkoti et al.[7] Stv-K127 was constructed to crosslink two Lys-127 residues across the dimer-dimer interface by using amino-specific homobifunctional crosslinkers. Stv-D127 was designed to make a hybrid streptavidin consisting of Stv-K127 and Stv-D127. This hybrid protein, if successfully produced, would have enhanced subunit association across the dimer-dimer interface because of the electrostatic interactions between the ε-amino group of Lys-127 of one subunit and the β-carboxyl group of Asp-127 of the adjacent subunit. This molecule should also allow the formation of an amide bond between these two residues by

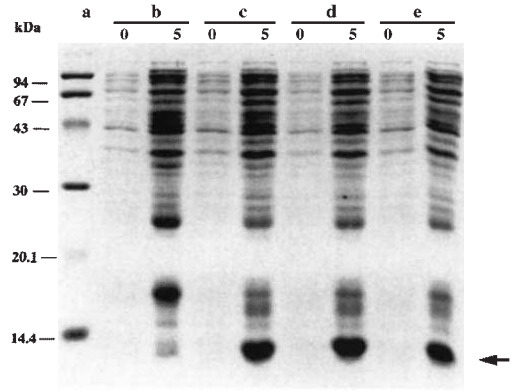

Figure 1. Expression of streptavidin mutants Stv-C127, Stv-D127, and Stv-K127. a, molecular mass standard proteins; b, BL21(DE3)(pLysE); c, BL21(DE3)(pLysE)(pTSA-C127); d, BL21(DE3)(pLysE)(pTSA-D127); and e, BL21(DE3)(pLysE)(pTSA-K127). Total cell protein at time 0 (the time of induction) (133 µl of culture) and 5 h after induction (67 µl of culture) was analyzed by SDS-PAGE. An arrow indicates the position where streptavidin mutants migrated. Samples were applied to gels after boiling for 2 min. Proteins were stained with Coomassie brilliant blue.

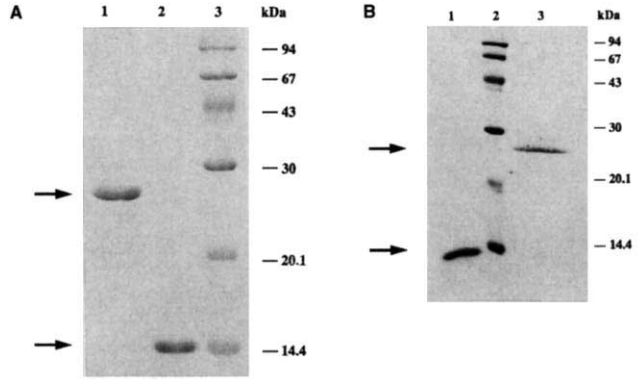

Figure 2. (A) SDS-PAGE analysis of disulfide bond formation in Stv-C127. Lane 1: Stv-C127 after removal of β-mercaptoethanol by lyophilization; lane 2: Stv-C127 in the presence of DTT; and lane 3: molecular mass standard proteins. (B) SDS-PAGE analysis of crosslinking of Stv-C127 with 1,3-dibromoacetone. Lane 1: Stv-C127 in the presence of DTT; lane 2: molecular mass standard proteins; and lane 3: Stv-C127 after crosslinking with 1,3-dibromoacetone. In both panels subunit dimers (top) and monomers (bottom) are shown by arrows. Samples were applied to gels after boiling for 2 min. Proteins were stained with Coomassie brilliant blue.

chemical crosslinking.

Expression and purification of streptavidin mutants. Each of Stv-C127, Stv-K127, and Stv-D127 was efficiently expressed in *Escherichia coli* (Fig. 1) using the T7 expression system[8]. Stv-C127 and Stv-K127 were purified to homogeneity[9] by a procedure that included 2-iminobiotin affinity chromatography[10]. Each of these two proteins formed a stable tetrameric molecule and had full biotin-binding ability. In contrast, Stv-D127 formed insoluble aggregates, suggesting that this protein alone cannot form a stable tetrameric molecule (Sano and Cantor, unpublished data).

Preparation of streptavidins with reversible and irreversible intersubunit crosslinks using Stv-C127. The cysteine residues at position 127 of Stv-C127 were oxidized by the removal of a reducing agent by lyophilization. Sodium dodecylsufate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 2A) demonstrates that sulfhydryl groups of adjacent subunits across the dimer-dimer interface were crosslinked at 100% efficiency (lane 1). Disulfide bonds were readily dissociated by the addition of DTT (dithiothreitol) (lane 2). Similar results were obtained with this protein after oxidation with hydrogen peroxide. The formation of a disulfide bond between two cysteine residues had no effect on the biotin-binding ability. The successful disulfide formation reveals that the two cysteine residues are located in close proximity across the dimer-dimer interface.

We also attempted to make an irreversible covalent bond between the two sulfhydryl groups through the dimer-dimer interface using the irreversible crosslinker 1,3-dibromoacetone. This compound reacts first with a sulfhydryl group and then with another nucleophile within a 5 Å radius[11,12]. SDS-PAGE analysis (Fig. 2B) shows the formation of crosslinked subunit dimers at >90% efficiency. The formation of an irreversible covalent bond between two sulfhydryl groups had no effect on the biotin-binding ability.

Preparation of a streptavidin with irreversible intersubunit crosslinks using Stv-K127. Stv-K127 was designed to make a crosslinked streptavidin in which an irreversible covalent bond is formed between the ϵ-amino groups of Lys-127 residues of adjacent subunits through the dimer-dimer interface. Several amino-specific homobifunctional crosslinkers were tested. SDS-PAGE of crosslinked products made by using bis(sulfosuccinimidyl)suberate (spacer = 11.4 Å; Pierce, Rockford, IL) showed several bands corresponding to subunit dimers, trimers, and tetramers, and higher molecular weight aggregates, indicating that both inter- and intramolecular crosslinking occurred. Amino-specific crosslinkers with shorter spacers, such as dimethyladipimidate-2HCl (spacer = 8.6 Å), disuccinimidyl glutarate (spacer = 7.7 Å), and *N*-hydroxysuccinimidyl 2,3-dibromopropionate (spacer = 5.0 Å; Pierce), were tested to see if nonspecific crosslinking reactions could be reduced. Although the formation of higher molecular weight aggregates was reduced, subunit trimers and tetramers were still formed, in addition to subunit dimers. These results indicate that Stv-K127 was unable to make specific crosslinks between Lys-127 residues through the dimer-dimer interface using the amino-specific homobifunctional crosslinkers tested.

Preparation of a hybrid streptavidin, Stv-DK127. Stv-D127 was used to make a hybrid tetrameric streptavidin consisting of Stv-K127 and Stv-D127. Stv-D127 alone forms insoluble aggregates and cannot make a stable tetramer. However, if a mixture of denatured monomeric Stv-D127 and Stv-K127 is renatured, a hybrid tetramer consisting of Stv-D127 and Stv-K127 might be preferentially formed over tetrameric Stv-K127. This might occur because such a hybrid streptavidin should have a more stable subunit association than tetrameric Stv-K127 due to the electrostatic attraction between the β-carboxyl group of Asp-127 of one subunit and the ϵ-amino group of Lys-127 of the adjacent subunit at the dimer-dimer interface. In practice, an excess Stv-D127 over Stv-K127 was used during renaturation to prevent the formation of Stv-K127 homotetramers.

To investigate if a hybrid tetramer had successfully been made, the purified protein was compared with Stv-K127 and natural core streptavidin (Boehringer Mannheim, Indianapolis, IN) by SDS-PAGE. These proteins remained tetrameric in the presence of SDS (Fig. 3), as shown previously for natural core streptavidin[13]. Comparison of the migration of these proteins indicates that the purified protein consists of a single species that migrates faster than Stv-K127 and natural core streptavidin. The faster migration of this purified protein suggests that it contains both Stv-D127 and Stv-K127 subunits.

Preparation of a hybrid streptavidin with irreversible intersubunit crosslinks using Stv-DK127. To confirm that the streptavidin construct, prepared from a mixture of Stv-D127 and Stv-K127, is a hybrid tetramer, we attempted to crosslink the carboxyl and amino side chains of amino acid 127 of adjacent

subunits by using the zero-length crosslinker EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Pierce). SDS-PAGE analysis shows that >80% of the subunits formed crosslinked dimers (Fig. 4, lane 5). Similar reactions were carried out on Stv-K127 homotetramers, but no such products were observed (Fig. 4, lane 2). The high efficiency of crosslinked dimer formation reveals that this streptavidin construct is a hybrid tetramer in which different subunits are positioned at the dimer-dimer interface at a ratio of Stv-D127:Stv-K127 of almost 1 (termed Stv-DK127). Tetramers, in which these two different subunits face each other at the dimer-dimer interface, should be electrostatically more stable than other possible combinations. Crosslinking of Stv-DK127 reduced its biotin-binding ability by approximately 10%, suggesting that other crosslinking reactions also occurred within the protein.

Recently, hybrid tetrameric streptavidins have been made by renaturating a mixture of two different denatured subunit species[7]. A denatured subunit dimer, connected via a disulfide bond between the two Cys-127 residues, which is very similar to Stv-C127 in this study, was used as one of the components to limit the subunit composition and configuration of the resulting tetramers. These methods generate mixed populations of tetrameric streptavidins, and thus, the purification of the hybrid tetramer species of interest from the others is needed. In contrast, Stv-DK127 can be self-assembled during renaturation in solution, leaving nonassociated Stv-D127 precipitated. Thus, a single hybrid tetramer species can be obtained without the need for further purification.

Biotinylated DNA-binding ability of streptavidins with intersubunit crosslinks. Although all of the crosslinked streptavidins are able to bind biotin, the introduction of covalent bonds across the dimer-dimer interface might affect the binding to larger biotinylated macromolecules if the intersubunit crosslinks reduced structural flexibility around the biotin-binding site. To test this, each crosslinked streptavidin was analyzed for its ability to bind a biotinylated 18-base DNA. Nondenaturing PAGE analysis of streptavidin-biotinylated DNA mixtures indicates that there is no apparent difference in the amounts of bound biotinylated DNA among the crosslinked streptavidins and natural core streptavidin (results not shown). This suggests that the introduction of covalent bonds through the dimer-dimer interface has little effect on the ability to bind biotinylated macromolecules.

Thermal stability of streptavidins with intersubunit crosslinks. All of the crosslinked streptavidins and natural core streptavidin were heated in the absence of biotin to see if the introduction of covalent bonds or the electrostatic interaction at the dimer-dimer interface could enhance the thermal stability of streptavidin. Each streptavidin construct was heated to temperatures up to 95°C, kept at these temperatures for 10 min, and cooled to 25°C. The resulting protein samples were subjected to SDS-PAGE analysis and biotin-binding assays.

After heat treatment streptavidin samples were applied to SDS-PAGE without boiling to maintain the tetrameric structure[13] (Fig. 5). Proteins were visualized by Coomassie brilliant blue or silver staining, and the amounts of tetrameric molecules were quantitated by densitometry (Fig. 5F). Most of natural core streptavidin (74%) dissociated into monomers at 80°C (Fig. 5A). In contrast, Stv-C127 with disulfide bonds maintained the tetrameric structure at 80°C (Fig. 5B). Even at 95°C, 26% of these molecules remained tetrameric. Stv-C127 with irreversible crosslinks remained tetrameric at 80°C (Fig. 5C). After heating to 90°C, however, a fraction of this construct dissociated into subunit dimers, and the remaining molecules formed aggregates. Uncrosslinked Stv-DK127 dissociated almost completely into monomers at 70°C (Fig. 5D), suggesting that the electrostatic attraction between the side chains of Lys-127 and Asp-127 at the dimer-dimer interface is not strong enough to maintain the tetrameric structure when

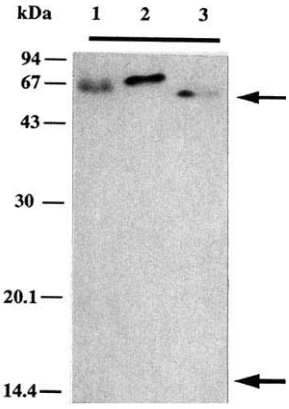

Figure 3. SDS-PAGE analysis of Stv-K127, Stv-DK127, and natural core streptavidin. Samples were applied to gels without boiling to maintain the tetrameric structure[13]. Lane 1: natural core streptavidin; lane 2: Stv-K127; and lane 3: Stv-DK127. Subunit tetramers (top) and monomers (bottom) are shown by arrows. Proteins were stained with Coomassie brilliant blue.

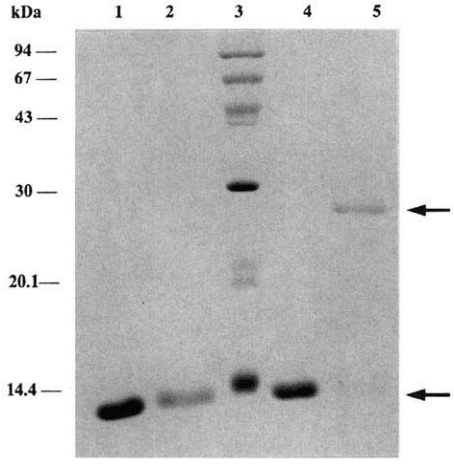

Figure 4. SDS-PAGE analysis of Stv-K127 and Stv-DK127 before and after crosslinking. Lane 1: uncrosslinked Stv-K127; lane 2: Stv-K127 after crosslinking with EDC; lane 3: molecular mass standard proteins; lane 4: uncrosslinked Stv-DK127; and lane 5: Stv-DK127 after crosslinking with EDC. Subunit dimers (top) and monomers (bottom) are shown by arrows. Samples were applied to gels after boiling for 2 min. Proteins were stained with Coomassie brilliant blue.

heated. Crosslinked Stv-DK127 (Fig. 5E) was more stable at 70°C than its uncrosslinked derivative, retaining >70% of its tetrameric structure at 70°C. The reduced amount of protein migrating into the gel above 25°C is caused by the formation of high molecular aggregates.

To determine the biotin-binding ability of streptavidin constructs after heat treatment, each streptavidin sample was mixed with excess D-[*carbonyl*-^{14}C]biotin. Unbound biotin, separated by filtration, was quantitated (Fig. 6). The biotin-binding ability of natural core streptavidin decreased to 55% when heated to 70°C. In contrast, streptavidins containing intersubunit crosslinks retained more than 60% of their original biotin-binding abilities when heated to 80°C. Uncrosslinked Stv-DK127 had only 29% of its biotin-binding ability when heated to 70°C.

These results indicate that the introduction of covalent bonds across the dimer-dimer interface enhances the thermal stability of streptavidin, making it more resistant to both subunit dissociation

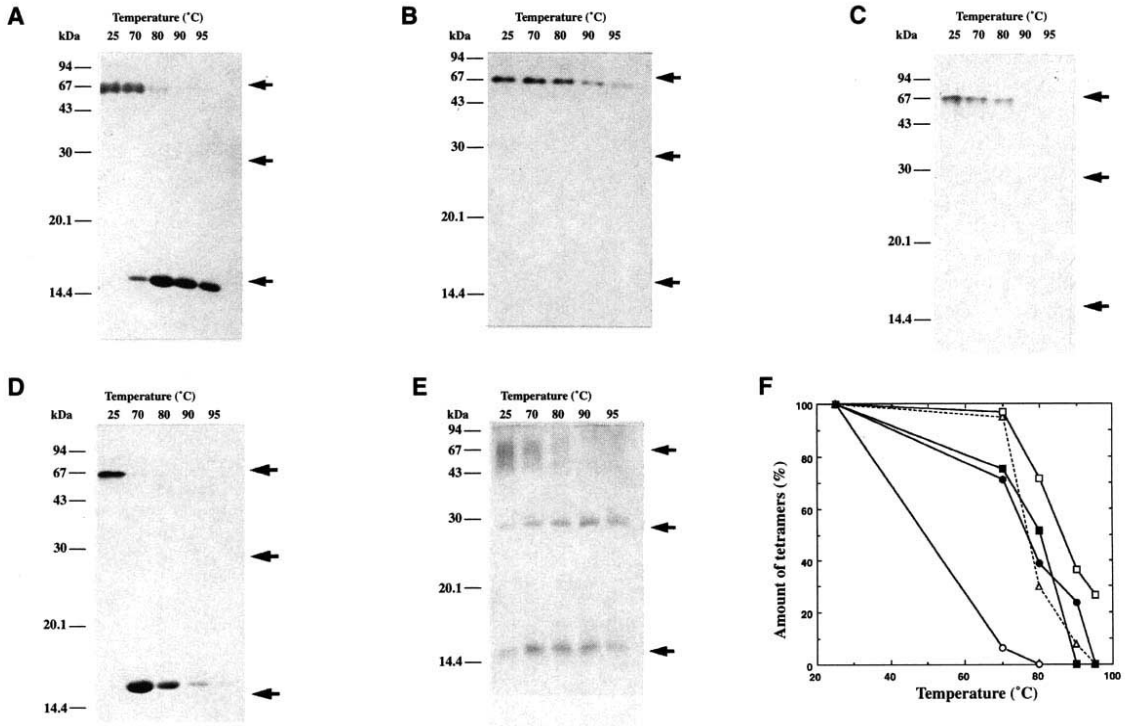

Figure 5. Thermal stability of streptavidins with intersubunit crosslinks, analyzed by SDS-PAGE. (A) natural core streptavidin; (B) Stv-C127 with disulfide bonds; (C) Stv-C127 crosslinked with 1,3-dibromoacetone; (D) uncrosslinked Stv-DK127; (E) Stv-DK127 crosslinked with EDC. Proteins were heated without biotin from 25°C at a constant rate of 2°C/min to temperatures indicated as described in the Experimental protocol. Subunit tetramers (top), dimers (middle), and monomers (bottom) are shown by arrows. Samples were applied to gels without boiling treatment to maintain the tetrameric structure[13]. (A–D) proteins were stained with Coomassie brilliant blue. (E) proteins were visualized by silver staining. The amounts of tetrameric molecules in each gel were quantitated by densitometry and are plotted as a function of temperature (F). Δ, natural core streptavidin; ❑, Stv-C127 with disulfide bonds; ■, Stv-C127 crosslinked with 1, 3-dibromoacetone; ○, uncrosslinked Stv-DK127; ●, crosslinked Stv-DK127.

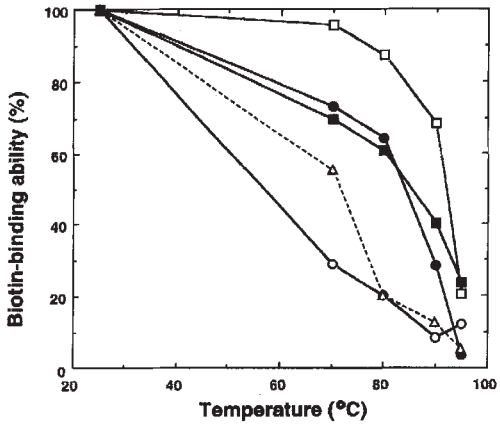

Figure 6. Thermal stability of streptavidins with intersubunit crosslinks, analyzed by biotin-binding assays. The remaining biotin-binding ability of each streptavidin construct after heat treatment was determined and is plotted as a function of temperature. Δ, natural core streptavidin; ❑, Stv-C127 with disulfide bonds; ■, Stv-C127 crosslinked with 1, 3-dibromoacetone; ○, uncrosslinked Stv-DK127; ●, crosslinked Stv-DK127.

and loss of biotin-binding ability. The amount of tetrameric molecules after heat treatment (Fig. 5F) shows a high correlation with biotin-binding ability (Fig. 6), suggesting that the maintenance of the tetrameric structure is essential for streptavidin to retain its biotin-binding ability.

Stability of streptavidins with intersubunit crosslinks in guanidine hydrochloride. We also tested the stability of each streptavidin construct against denaturation by guanidine hydrochloride. The ability of these proteins to retain bound biotin was used as a measure of stability. Each streptavidin construct was saturated with biotin and incubated in 7 M guanidine hydrochloride (pH 0.89). The amount of free, released biotin was quantitated after separation from the streptavidin-bound biotin.

Natural core streptavidin was least stable among the molecules tested, retaining only 35% of bound biotin. Stv-C127 with disulfide bonds across the dimer-dimer interface retained 40% of bound biotin, whereas Stv-C127 with irreversible covalent bonds retained approximately 48% of bound biotin. Uncrosslinked Stv-DK127 and its crosslinked derivative were the most stable of the five protein species tested, retaining 70% of bound biotin.

Conclusions. We have shown that the introduction of covalent bonds between adjacent subunits across the dimer-dimer interface enhances the overall stability of streptavidin with little effect on its biotin-binding ability. The enhanced ability of the crosslinked streptavidins to retain bound biotin suggests that the disruption of the streptavidin molecule along the weak dimer-dimer interface is one of the factors that causes the release of biotin from streptavidin. These crosslinked streptavidins should also be useful in bioanalytical applications. For example, immobilization of these proteins on solid surfaces would provide more stable binding of biotinylated macromolecules, because the enhanced subunit association of the crosslinked streptavidins would prevent their release. These streptavidin constructs could also be used in assays requiring high temperatures, such as in the polymerase chain reaction in which these proteins would remain bound to biotinylated DNA molecules without denaturation even at temperatures used for dissociation of double-stranded DNA.

Experimental protocol

Construction of expression vectors. Expression vectors were constructed by using a bacteriophage M13mp18 derivative, mpSA-29, which codes for a core streptavidin consisting of amino acids 16 to 133, as a starting material. Mutations were introduced into the coding sequence for streptavidin by using an oligonucleotide-directed in vitro mutagenesis system (Amersham, Arlington Heights, IL)[15]. The following three sets of mutations were made separately on the codon for His-127 (CAC): TGC for Cys; AAA for Lys; and GAC for Asp. The coding sequence containing the desired mutations was cloned into the NdeI site of plasmid pET-3a under the control of the *ϕ10* promoter[8]. The resulting expression vectors, pTSA-C127, pTSA-K127, and pTSA-33, encode the streptavidin mutants Stv-C127, Stv-K127, and Stv-D127, in which His-127 is replaced by Cys, Lys, and Asp, respectively.

Expression and purification of streptavidin mutants. Expression and purification of each streptavidin mutant was carried out as previously described[9,10] by using *E. coli* strain BL21(DE3)(pLysE)[8] carrying an expression vector. For Stv-C127, β-mercaptoethanol was included in all solutions to prevent disulfide bond formation during purification[16].

Preparation of a streptavidin with reversible intersubunit crosslinks using Stv-C127. Disulfide bonds between the sulfhydryl groups of Cys-127 residues across the dimer-dimer interface were formed by lyophilizing Stv-C127 to remove β-mercaptoethanol used to prevent disulfide bond formation during purification. Disulfide bonds were also formed by treating the protein with 0.15% hydrogen peroxide at room temperature (~22°C) for 90 min. After disulfide bond formation, proteins were dialyzed against water and stored at 4°C.

Preparation of a streptavidin with irreversible intersubunit crosslinks using Stv-C127. The crosslinker 1,3-dibromoacetone (ICN, Costa Mesa, CA) was used to make an irreversible covalent bond between the cysteine residues of Stv-C127. Lyophilized Stv-C127 (3 μg) was dissolved in 6 μl of 5 mM DTT, 100 mM potassium phosphate (pH 7.8). After incubation at room temperature for 1 h, the DTT concentration was reduced to 1 mM by the addition of 100 mM potassium phosphate (pH 7.8). Then, 10 μl of 1,3-dibromoacetone dissolved in ethanol was added to a final concentration of 2 mM. The reaction mixture was incubated at room temperature for 15 min in the dark, and then the reaction was terminated by the addition of DTT to a final concentration of 5 mM. The resulting proteins were dialyzed against water and stored at 4°C.

Preparation of a hybrid streptavidin with irreversible intersubunit crosslinks using Stv-D127 and Stv-K127. Preparation of a hybrid tetrameric streptavidin was attempted by mixing crude Stv-D127 and Stv-K127 in 7 M guanidine hydrochloride (pH 1.5) at an approximately 4:1 ratio, followed by renaturation by removal of guanidine hydrochloride. The subsequent purification procedure was the same as described above. Purified protein was dialyzed against water and stored at 4°C. A zero-length crosslinker, EDC, was used to crosslink the β-carboxyl group of Asp-127 of one subunit with the ϵ-amino group of Lys-127 of an adjacent subunit. Sulfo-*N*-hydroxysuccinimide (Pierce) was used to improve the conjugation efficiency[17]. Approximately 60 pmol (240 pmol of subunits) of lyophilized Stv-DK127 were dissolved in 100 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.0), 5 mM sulfo-*N*-hydroxysuccinimide, 30 mM EDC to initiate crosslinking reactions. Relatively low protein concentrations were used to minimize aggregate formation caused by intermolecular crosslinking. The reaction mixture was incubated at room temperature for 3 h, and then 50 mM hydroxylamine HCl was added to quench the reaction and to regenerate unreacted carboxyl groups. The crosslinked protein was dialyzed against water and stored at 4°C.

Thermal stability of streptavidins with intersubunit crosslinks. Each streptavidin construct (approximately 240 pmol of subunits), dissolved in 6.5 μl of water, was heated from 25°C to 70°C, 80°C, 90°C, or 95°C at a rate of 2°C/min. The protein solutions were kept at these temperatures for 10 min and then cooled to 25°C at a rate of 2°C/min. The resulting protein samples were subjected to SDS-PAGE analysis and biotin-binding assays. For SDS-PAGE analysis, protein samples were incubated at room temperature for 1 h in 1.25% SDS, 40 mM Tris-HCl (pH 6.8), and then analyzed by SDS-PAGE without boiling. Proteins were visualized with Coomassie brilliant blue or silver staining, and the amounts of tetrameric streptavidins were quantitated by densitometry (420 oe; PDI, Inc., Huntington Station, NY) with the software package Quantity One V. 2.5 (PDI, Inc.). To determine the biotin binding ability of each streptavidin construct after heat treatment, D-[*carbonyl*-^{14}C]biotin was added to each streptavidin sample at a ratio of biotin:biotin-binding site of 1.4 in 150 mM NaCl, 50 mM Tris-Cl (pH 7.5). After the mixtures were incubated at room temperature for 30 min, unbound biotin was separated from streptavidin-biotin complexes using Ultrafree-MC centrifugal filter units (molecular mass cutoff, 10 kD; Millipore, Bedford, MA) and quantitated by liquid scintillation counting.

Stability of streptavidins with intersubunit crosslinks in guanidine hydrochloride. Each streptavidin construct (approximately 160 pmol of subunits) was incubated for 20 min in 15 μl of D-[8,9-^{3}H]biotin (47 Ci/mmol; Amersham) to fill approximately 22 pmol of the biotin-binding site, followed by the addition of 2 μl of unlabeled biotin (145 pmol) to fill the remaining biotin-binding sites. Then, 983 μl of 7 M guanidine hydrochloride (pH 0.89) was added, and the mixture was incubated at room temperature for 90 min. Control experiments were performed by incubating the proteins in 150 mM NaCl, 50 mM ammonium acetate (pH 6.0) without guanidine hydrochloride. Released biotin was separated as above.

Biotinylated DNA-binding ability of streptavidin mutants. Each lyophilized streptavidin construct was mixed with an end-biotinylated 18-base DNA, prepared using an automated DNA synthesizer, in 4.5 M NaCl at a 1:100 molar ratio, and the mixture was incubated at room temperature for 2 h. The mixture was desalted by filtration and analyzed on a 15% polyacrylamide gel under nondenaturing conditions[18].

Other methods. SDS-PAGE analysis[19] was carried out using 15% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue or by silver staining (Bio-Rad, Hercules, CA). Biotin-binding ability was determined by gel filtration chromatography[20] using PD-10 columns (Pharmacia, Piscataway, NJ) and D-[*carbonyl*-^{14}C]biotin.

Acknowledgments

This work was supported by a grant (DE-FG02-93ER61656) from the US Department of Energy.

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