Engineering subunit association of multisubunit proteins: A dimeric streptavidin

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ABSTRACT A dimeric streptavidin has been designed by molecular modeling using effective binding free energy calculations that decompose the binding free energy into electrostatic, desolvation, and side chain entropy loss terms. A histidine-127 → aspartic acid (H127D) mutation was sufficient to introduce electrostatic repulsion between subunits that prevents the formation of the natural tetramer. However, the high hydrophobicity of the dimer–dimer interface, which would be exposed to solvent in a dimeric streptavidin, suggests that the resulting molecule would have very low solubility in aqueous media. In agreement with the calculations, a streptavidin containing the H127D mutation formed insoluble aggregates. Thus, the major design goal was to reduce the hydrophobicity of the dimer–dimer interface while maintaining the fundamental structure. Free energy calculations suggested that the hydrophobicity of the dimer–dimer interface could be reduced significantly by deleting a loop from G113 through W120 that should have no apparent contact with biotin in a dimeric molecule. The resulting protein, containing both the H127D mutation and the loop deletion, formed a soluble dimeric streptavidin in the presence of biotin.

There are many examples of the successful production of individual subunits of multisubunit proteins using recombinant DNA technology. However, in most of these successful cases, each subunit functions independently of the other subunits in its parental multisubunit protein. Thus, the separation of subunits does not directly affect the fundamental function and structure of the subunit. A well studied example is recombinant antibodies (IgGs) (1–7). The disulfide bonds and noncovalent interactions between the two heavy chains of an IgG and those between the constant regions of the heavy chain and the light chain have little effect on the folding and function of the antigen-binding site consisting of the variable regions of the heavy and light chains. Thus, the heavy chain and light chain can be expressed separately, and they can be assembled to form the parental bivalent antibody. A fragment of the heavy chain, in which the cysteine residues making the inter-heavy chain disulfide bonds are truncated but the cysteine residue making the heavy chain–light chain disulfide bond is present, is still capable of making a disulfide bond with the light chain, forming a monovalent antibody (Fab fragment). In addition, the variable regions of the heavy and light chains without the interchain disulfide bond can even be fused recombinantly with an appropriate linker, resulting in the formation of a single-chain antibody. In contrast, protein engineering of individual subunits of multisubunit proteins, in which subunit association is essential for their functionality and structure, has had limited success (8–13). This is because disruption of the subunit–subunit interactions reduces the ability to fold and maintain the functional three-dimensional

structure. In streptavidin, a tetrameric protein produced by *Streptomyces avidinii* (14, 15), the subunit association is essential for both the extremely tight biotin binding and structural stability.

In this work, we attempted to construct a dimeric streptavidin, as opposed to the natural tetrameric structure, by disrupting the subunit association without disturbing the basic structure of each subunit. The design of a dimeric streptavidin requires that the following three well defined problems be addressed; similar problems generally occur when reducing the size of multisubunit proteins. First, the tetramer formation must be prevented, preferably by introducing a minimum number of mutations. Second, the separation of a tetramer into dimers exposes to solvent the side chains of a large number of hydrophobic amino acid residues, located at the subunit–subunit interface in tetrameric streptavidin. Thus, a dimeric streptavidin with sufficient solubility in aqueous media can only be formed by substantially reducing the hydrophobicity of the exposed subunit–subunit interface. Third, because the intersubunit contact made by tryptophan-120 (W120) to biotin bound by an adjacent subunit significantly contributes to biotin binding by tetrameric streptavidin (16–19), the biotin-binding affinity of a dimeric streptavidin is likely to be reduced. The first two problems should be addressable by molecular calculations using an enhanced empirical binding free energy evaluation model (20–22). This model, developed for the analysis of complexes, assumes, as a first-order approximation, rigid body association. The same binding free energy evaluation model could also be used to address the third problem for further design of dimeric streptavidins, in which the biotin-binding affinity, reduced due to the subunit separation, is restored.

The present study focused on the first two problems. The binding free energy evaluation model was applied to these problems to see if the first generation of dimeric streptavidins could be designed. The resulting dimeric streptavidins were produced and characterized experimentally to see how well the molecular modeling approach used can predict their properties. This study is expected to provide useful guidelines for the design of further generations of reduced-size streptavidins with the properties equivalent to tetrameric streptavidin, including the biotin-binding ability, by addressing the third and possibly additional problems.

MATERIALS AND METHODS

Molecular Modeling and Free Energy Calculations. An empirical method of binding free energy calculation for reactions, in which the conformation of the reactants remains nearly unchanged, was used (20–22). The binding free energy, ΔG, is decomposed according to the equation $\Delta G = E_{el} + \Delta G_d - T\Delta S_{sc} + \Delta G_{const}$, where E_{el} is the electrostatic interaction

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energy between the two reactant molecules, ΔG_d is the desolvation contribution to the binding free energy, and $T\Delta S_{sc}$ denotes the entropic term associated with the conformational entropy loss of the side chains of the reactants upon association. The last term, ΔG_{const}, includes rotational, translational, and cratic free energies, and, as a first-order approximation, it was assumed to be constant at 11 kcal/mol.

The electrostatic interaction energy E_{el} is calculated by the Coulombic expression using a molecular mechanics potential function CHARMM 19 (23) with the distance-dependent dielectric permittivity $\varepsilon = 4r$. The desolvation term, ΔG_d, is estimated by an atomic solvation parameter model (24) as a linear combination of the accessible areas of different atoms using an updated set of solvation parameters (20). The desolvation effect usually includes large negative hydrophobic contributions associated with the removal of non-polar atoms from water and smaller positive terms due to the desolvation of polar and charged atoms that become buried upon association of the reactants (21). The loss of side chain conformational entropy, ΔS_{sc}, is estimated by using an empirical entropy scale (20).

The binding free energy was calculated for natural streptavidin using the known three-dimensional structure (16, 17). The models of streptavidin variants (Stv-33 and Stv-43) were built with the help of the QUANTA program (Molecular Simulations, Waltham, MA). The free energy model assumes good packing of both protein–protein and protein–solvent interfaces as seen in the x-ray structures of protein–protein complexes. Therefore, before free energy evaluation, the steric clashes were removed by subjecting the complex to energy minimization using the CHARMM potential.

Construction of Expression Vectors. Expression vectors were constructed by standard techniques (25) using pTSA-13 (26), which encodes the minimum-sized core streptavidin consisting of amino acid residues 16–133 (27), as a starting material.

Oligonucleotide-directed *in vitro* mutagenesis was performed on a bacteriophage M13 mp18 derivative carrying the entire coding sequence for the minimum-sized core streptavidin to convert the codon for H127 (CAC) to GAC for D (H127D) using an 18-base oligonucleotide, 5′-d(AGGTG TCGTC GCCGA CCA)-3′. The resulting coding sequence was cloned into the *Nde*I site of the plasmid pET-3a under the Φ*10* promoter (28). The resulting expression vector, pTSA-33, encodes a core streptavidin mutant, Stv-33 (12.6 kDa per subunit), which contains the H127D mutation.

Two separate PCR amplifications were performed using pTSA-33 as the template to generate two partial DNA fragments of the streptavidin coding sequence. One PCR amplification used the following primers: 5′-AATAC GACTC AC-TAT AG-3′ and 5′-GTTG̲T̲ T̲CGAA GTCAG CAGCC AC-TGG GT-3′. This PCR amplification generated a 380-bp fragment containing a sequence from the translation initiation site to the codon for S112, followed by the *Bst*BI recognition sequence (underlined). The other PCR amplification used the following primers: 5′-TTGC̲T̲ T̲CGAA GTCCA CGCTG GTCGG C-3′ and 5′-CGGGC TTTGT TAGCA GCCGG A-3′. This PCR amplification generated a 150-bp fragment containing a *Bst*BI recognition site (underlined), followed by the sequence from the codon for K121 to a translation termination codon. The 380-bp fragment was digested with *Nde*I and *Bst*BI, and the 150-bp fragment was digested with *Bam*HI and *Bst*BI. The resulting two fragments were ligated via the *Bst*BI termini (420 bp) and cloned between the *Nde*I and *Bam*HI sites of pET-3a under the Φ*10* promoter. The resulting expression vector, pTSA-43, encodes a core streptavidin mutant, Stv-43 (11.8 kDa per subunit), in which the sequence from G113 through W120 has been deleted in addition to the H127D mutation. pTSA-43 also carries silent

mutations in the codons for T111 (ACC to ACT) and S112 (TCC to TCG).

Expression of Dimeric Streptavidins. Each streptavidin mutant was expressed in *Escherichia coli* using the T7 expression system (28) by the method previously described (26, 29, 30). Briefly, *E. coli* strain BL21(DE3)(pLysE) carrying an expression vector was grown at 37°C with shaking in Luria–Bertani medium supplemented with 0.4% glucose, 150 μg/ml ampicillin, and 25 μg/ml chloramphenicol. When the absorbance at 600 nm of the culture reached 0.6, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce the expression of the T7 RNA polymerase gene placed under the control of the *lac*UV5 promoter. After induction, cells were incubated at 37°C with shaking.

Purification of Dimeric Streptavidins. Preparation of crude Stv-33 was carried out by the method used for other recombinant streptavidin derivatives (26, 29, 30). Briefly, a cell lysate of BL21(DE3)(pLysE) carrying pTSA-33, which had been incubated for 4 hr after induction, was prepared and then treated with DNase I and RNase A. An inclusion body (insoluble) fraction was collected by centrifugation of the cell lysate and dissolved in 7 M guanidine hydrochloride, pH 1.5. The dissolved protein solution was dialyzed against 0.2 M ammonium acetate, pH 6.0/0.02% Tween 20/0.02% NaN$_3$, to remove guanidine hydrochloride. The dialyzed fraction was centrifuged at 39,000 × g for 20 min, and the supernatant was used as crude Stv-33.

To purify Stv-43, several modifications were made to the procedure used for Stv-33. An inclusion body fraction was prepared by the method described above from BL21(DE3)-(pLysE) carrying pTSA-43, which had been incubated for 4 hr after induction, and dissolved in 7 M guanidine hydrochloride, pH 1.5. To the resulting solution, biotin was added to a final concentration of 2.4 μg/ml (10 μM), and the mixture was dialyzed against 0.2 M ammonium acetate, pH 6.0/0.02% Tween 20/0.02% NaN$_3$, containing 2.4 μg/ml biotin, and then against Tris-buffered saline containing Tween 20 (TTBS; 150 mM NaCl/20 mM Tris·Cl, pH 7.4/0.02% NaN$_3$/0.02% Tween 20) plus 2.4 μg/ml biotin. The dialyzed fraction was centrifuged at 39,000 × g for 20 min, and the supernatant was filtered through a 0.22-μm cellulose acetate membrane filtration unit (Falcon 7111). To remove biotin bound to Stv-43, the filtrate (100 ml) was subjected to three sequential dialysis steps: first against TTBS (two to three times, each with 500 ml for ≈1 hr) to remove free, unbound biotin; second against TTBS containing 10 mM urea (twice, each with 2,000 ml for several hours) to remove bound biotin; and third against TTBS (several times, each with 500 ml for ≈2 hr) to remove urea. The dialyzed fraction was applied to a biotin-agarose (Pierce) column, which had been equilibrated with TTBS. Unbound materials were removed by washing the column with TTBS, and bound proteins were eluted with TTBS containing 700 mM urea and 24 μg/ml biotin. The eluted proteins were dialyzed against TTBS containing 2.4 μg/ml biotin to remove urea, and the dialyzed fraction was filtered through a 0.22-μm poly(vinylidene fluoride) membrane filtration unit (Millex-GV; Millipore). The filtrate, containing purified Stv-43 with biotin, was stored at 4°C.

To remove bound biotin from purified Stv-43, the protein was subjected to the three sequential dialysis steps used during the purification of Stv-43. The resulting Stv-43 free from biotin was stored at 4°C and used within 3 days after preparation.

Biotin-Binding Ability. Purified Stv-43 without biotin (approximately 10 μg, 850-pmol subunits) was mixed with 10 nmol of D-[*carbonyl*-^{14}C]biotin (53 mCi/mmol; Amersham; 1 Ci = 37 GBq), and the mixture was incubated at 4°C for 30 min. The mixture was transferred to an Ultrafree-MC centrifugal filtration unit (molecular mass cut-off, 10 kDa; Millipore) and centrifuged at 5,000 × g for 15 min. The amount of biotin bound to Stv-43 was estimated from the difference in radio-

activity, determined by liquid scintillation counting, between the total D-[*carbonyl*-^{14}C]biotin used in the assay and unbound biotin in the filtrate fraction.

Biotin-Binding Affinity. Purified Stv-43 without biotin (final concentration, 0.56 μg/ml, 43 nM subunits) was mixed in TTBS (total volume, 200 μl) with various amounts of D-[8,9-^{3}H]biotin (46 Ci/mmol; Amersham; final concentration, 48.8 nM to 4.88 μM), and the mixture was incubated at 4°C for 24 hr. Free, unbound biotin was separated from streptavidin–biotin complexes by using Ultrafree-MC centrifugal filtration units that were centrifuged at 4°C at $5,000 \times g$ for 10 min. The amounts of biotin used in each mixture and in the filtrate were measured by liquid scintillation counting to determine the concentrations of streptavidin–biotin complexes and free biotin, from which the biotin-binding affinity constant (K_a) was estimated using Scatchard plots.

In this procedure, the concentrations of streptavidin and biotin change during the centrifugal filtration used to separate free biotin from streptavidin–biotin complexes; this shifts the equilibrium between streptavidin and biotin. Thus, potential errors are introduced into the binding affinity constants de-termined. However, when the affinity constant is relatively high, such errors are sufficiently small to neglect.

Other Methods. Gel filtration chromatography was per-formed by using a Superdex 75 HR 10/30 column with a fast protein liquid chromatography system (Pharmacia). Molec-ular mass standard proteins (Boehringer Mannheim) and the minimum-sized core streptavidin Stv-13 (50.4 kDa) (26) were used for calibration. Detailed conditions are give in the legend to Fig. 3. SDS/PAGE (31) was performed with use of 16% polyacrylamide gels. Proteins were stained with Coo-massie brilliant blue. The concentration of Stv-43 was deter-mined spectrophotometrically using an extinction coefficient $E_{280\ nm}^{0.1\%} = 3.25$, which was estimated from those of natural core streptavidin (15) and a streptavidin mutant containing a W120F mutation (18).

RESULTS AND DISCUSSION

Design of Dimeric Streptavidins. The x-ray structure of natural tetrameric streptavidin (16, 17) shows that each mol-ecule has two subunit interfaces (Fig. 1*A*). There is a strong
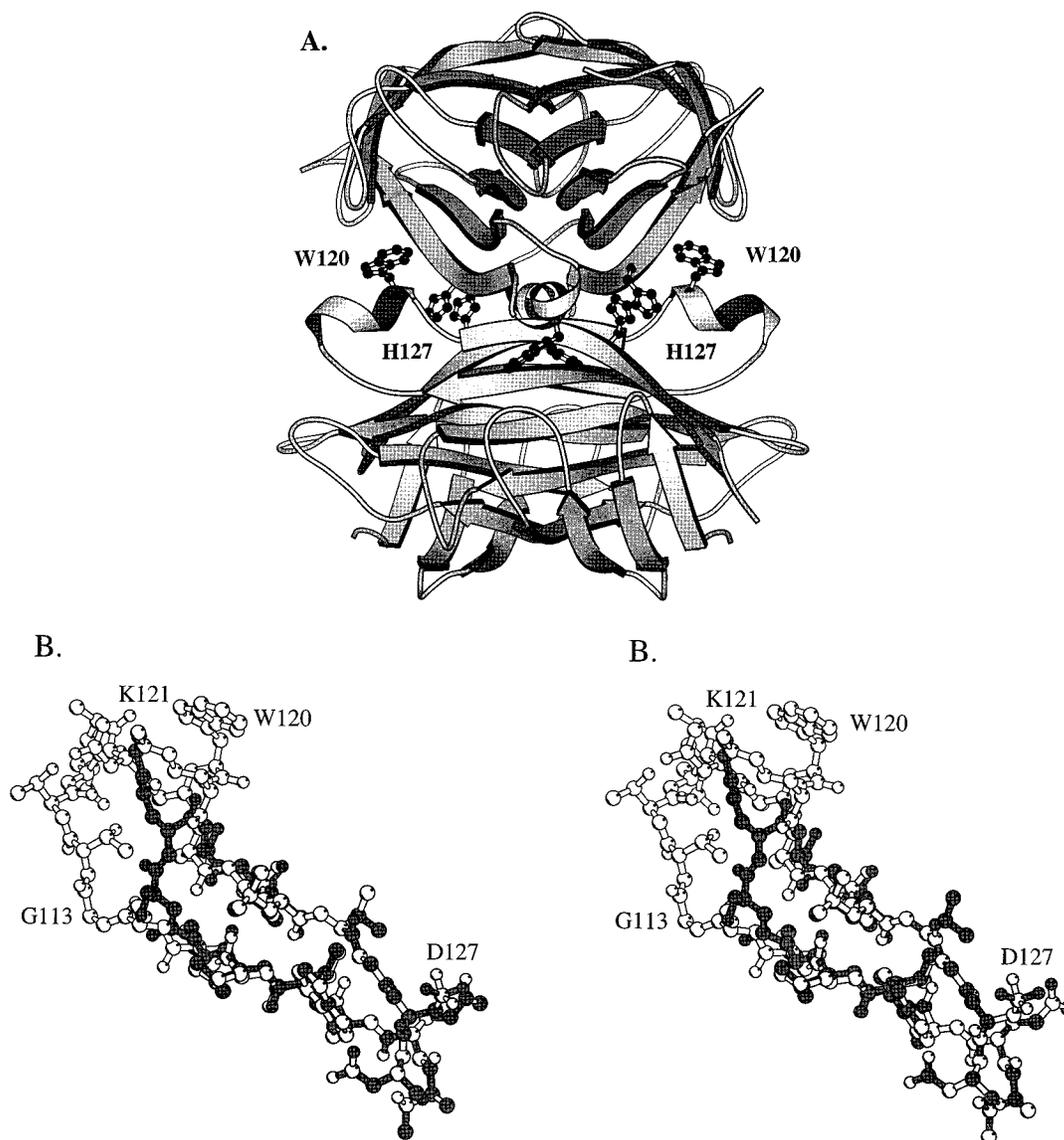
FIG. 1.   (*A*) Backbone structure of a tetrameric streptavidin. Also shown are the side chains of W120 and H127; the latter is converted to D127 in Stv-33 and Stv-43. (*B*) Stereo view of the local three-dimensional structure (T106–D128) around the loop region (G113–W120) which has been deleted in Stv-43 (shaded), superimposed on the same sequence of natural streptavidin. These are drawn based on the x-ray structure of natural streptavidin (16, 17) by using MOLSCRIPT (32).

interface (monomer–monomer interface) between a pair of subunits (stable dimer) that are associated tightly. The subunit barrel surfaces have complementary curvatures, making numerous intersubunit van der Waals and electrostatic interactions. The tetramer is formed by the association of two such stable dimers, which are connected relatively weakly across a small intersubunit contact area. The difference in calculated stability between these two subunit interfaces (see Tables 1 and 2) suggests that it should be easier to design dimeric streptavidins by disrupting the subunit association at the weaker dimer–dimer interface. Disruption of the subunit association at the dimer–dimer interface should form a globular dimeric protein, in contrast to a highly extended dimeric molecule formed by disruption at the monomer–monomer interface. Thus, it should expose to solvent a smaller number of hydrophobic amino acid residues that are located at the subunit–subunit interface in tetrameric streptavidin.

In the known three-dimensional structure of natural streptavidin (16, 17), the side chains of the H127 residues from adjacent subunits are in close proximity (3.1 Å) across the dimer–dimer interface. This has been shown by making a tetrameric streptavidin mutant containing C127, in place of H127, in which disulfide bonds are successfully formed between two pairs of the C127 residues through the dimer–dimer interface (33, 34). Replacing H127 with a charged amino acid ought to prevent two stable dimers from associating with each other through the dimer–dimer interface because of electrostatic repulsion between the two charged amino acid residues at the interface.

A streptavidin mutant (Stv-33) containing an H127D mutation should have a highly unfavorable electrostatic interaction energy between subunits at the dimer–dimer interface (E_{el} = 14.6 kcal/mol; Table 1). However, the major contribution to the binding free energy at the dimer–dimer interface of natural streptavidin is derived from the highly favorable desolvation term ΔG_d (−68.9 kcal/mol), which is almost unaffected by the H127D mutation (−64.2 kcal/mol). Because typical ΔG_d values of protein–protein complexes, such as protease–protein inhibitor complexes, are in a range from around −20 to −30 kcal/mol (20), the highly hydrophobic dimer–dimer interface (low ΔG_d), which is exposed to solvent in a dimeric streptavidin, would provide Stv-33 with very limited solubility in aqueous media.

Several design strategies can be used to improve the solubility characteristics of Stv-33. The most conservative strategy is to replace the hydrophobic residues in the dimer–dimer interface region with polar or charged amino acids. The most hydrophobic side chain that is exposed to solvent in a dimeric molecule is W120. In natural streptavidin, the side chain of W120 makes intersubunit contacts to biotin bound by an adjacent subunit through the dimer–dimer interface (16, 17). In a dimeric streptavidin, the latter subunit is no longer present, and W120 should be fully exposed to solvent. However, replacing W120 with a polar or charged residue, in addition to the H127D mutation, will not reduce the hydrophobicity to the level needed for sufficient solubility. For example, the W120E mutation should raise the ΔG_d to −42.7

kcal/mol, but this may not be high enough to provide the resulting protein with sufficient solubility. Free energy calculations reveal that at least two more hydrophobic side chains, L124 and V125, must be replaced, but even this would provide only limited solubility.

Here, a different, drastic strategy was used, in which the entire loop region from G113 through W120 that should have no apparent contact with biotin in a dimeric molecule, is truncated in addition to the H127D mutation (Fig. 1*B*). S112 and K121 are located at the termini of the seventh and eighth β-strands, respectively, and the carbonyl carbon of S112 and the amide nitrogen of K121 are 9 Å apart. This gap should be closable by adjusting the conformations of these two residues, placing a β-turn between the seventh and eighth β-strands. Thus, deletion of the loop by fusing S112 to K121 should have little effect on the subunit structure. Placing a β-turn should fuse the β-strands with minimal structural perturbation. The resulting molecule (Stv-43), which has both the H127D mutation and the deletion of the G113–W120 loop, should still
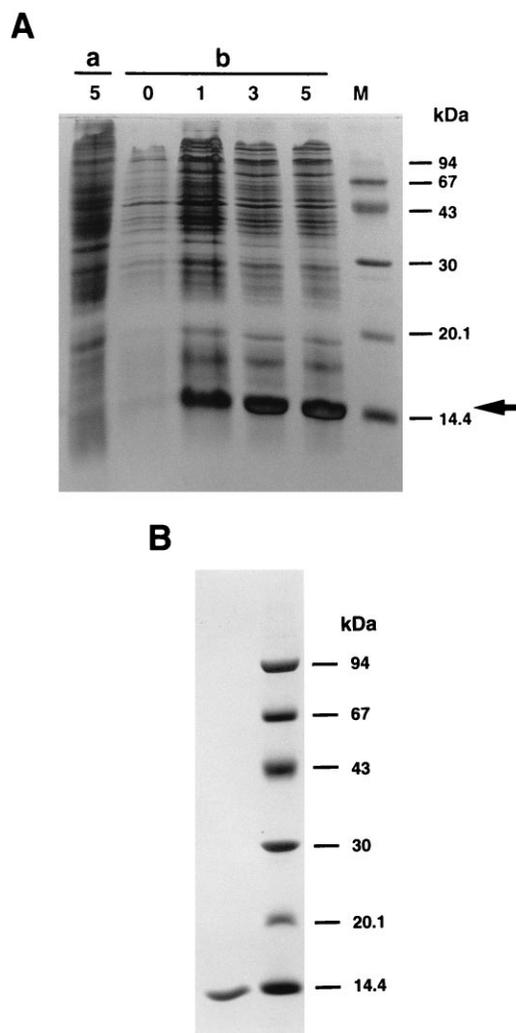
FIG. 2. (*A*) Expression of Stv-43 in *E. coli* carrying expression vector pTSA-43. Total cell protein of BL21(DE3)(pLysE), with or without pTSA-43, was analyzed by SDS/PAGE (31). Lanes: a, BL21(DE3)(pLysE); b, BL21(DE3)(pLysE)(pTSA-43); and M, molecular mass standard proteins. The number above each lane is the time in hours after induction. The position where Stv-43 migrates is shown by an arrow. Each lane contains the total cell protein from the following volume of culture: at 5 hr for a and at 3 hr and 5 hr for b, 25 µl; at 0 hr and 1 hr for b, 83 µl. (*B*) SDS/PAGE analysis of purified Stv-43. Approximately 1 µg of purified Stv-43 was analyzed. The right lane contains molecular mass standard proteins.

Table 1. Subunit–subunit interaction free energies through the dimer–dimer interface in natural tetrameric streptavidin and its dimeric mutants

Free energy term	Natural streptavidin	Stv-33	Stv-43
Electrostatic (E_{el})	−5.8	14.6	8.4
Desolvation (ΔG_d)	−68.9	−64.2	−23.4
Entropic ($-T\Delta S_{sc}$)	27.9	27.1	5.6
Others (ΔG_{const})	11.0	11.0	11.0
Total	−35.8	−11.5	1.6

All values are expressed as kilocalories per mole.
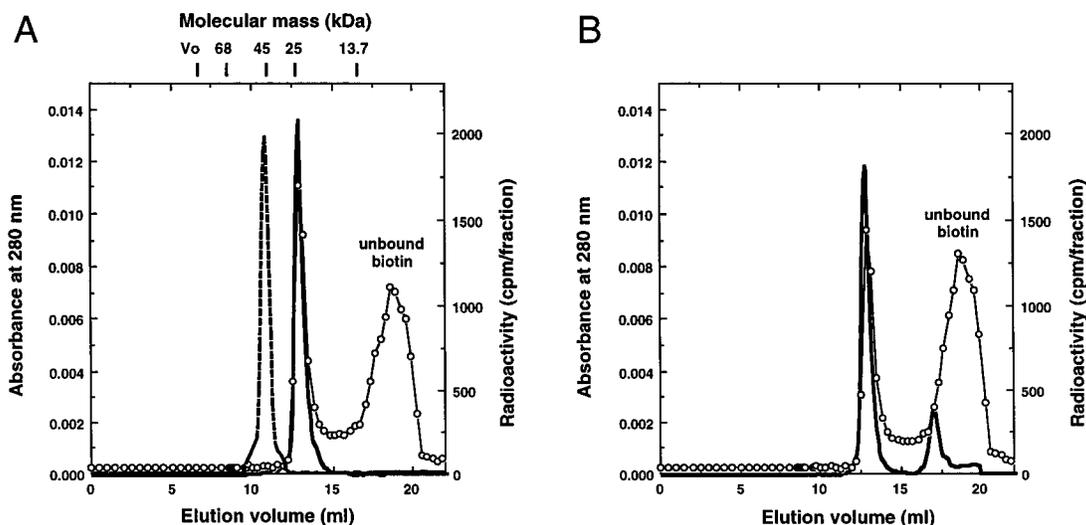
FIG. 3. Gel filtration chromatography of Stv-43. (*A*) Purified Stv-43 (approximately 3 μg) with excess D-[*carbonyl*-^{14}C]biotin was applied to a Superdex 75 HR 10/30 column (1.0 × 30 cm) that had been equilibrated with TTBS. Proteins were eluted at room temperature (22°C) with TTBS, and the absorbance at 280 nm was monitored (solid line). The eluate was collected in 320-μl fractions, and the radioactivity of each fraction was determined (○). The minimum-sized core streptavidin (50.4 kDa) (26) was also analyzed, and the absorbance at 280 nm is shown by a dashed line. (*B*) Stv-43, which had been stored without bound biotin at 4°C for 7 days, was mixed with excess D-[*carbonyl*-^{14}C]biotin. The mixture was analyzed by gel filtration chromatography by the same procedure as in *A*.

maintain a positive E_{el} for association between the two dimers via the dimer–dimer interface (8.4 kcal/mol; Table 1). However, its ΔG_d of the dimer–dimer interface region (−23.4 kcal/mol) is considerably higher than those of Stv-33 (−64.2 kcal/mol) and natural streptavidin (−68.9 kcal/mol), suggesting that this molecule could maintain a dimeric structure with sufficient solubility in aqueous media.

Expression and Purification of a Streptavidin Mutant, Stv-33. The streptavidin mutant Stv-33, which has the H127D mutation, was expressed efficiently in *E. coli* using the T7 expression system (28) (data not shown). Attempts were made to purify expressed Stv-33 by the methods used successfully for other streptavidins (26, 29, 30). However, Stv-33 formed insoluble aggregates upon renaturation of denatured inclusion bodies. Similar results were obtained when several different renaturation conditions were tested or when biotin was included in the renaturation solution.

This result is in good agreement with the binding free energy calculations described above. Stv-33 should have the highly hydrophobic dimer–dimer interface exposed to solvent, and thus it should have very limited solubility in aqueous media. This explains why Stv-33 forms insoluble aggregates upon renaturation. Thus, although the H127D mutation can prevent the association of two dimers through the dimer–dimer interface, it is unable to produce a dimeric streptavidin with sufficient solubility in aqueous media. The major obstacle in making a functional dimeric streptavidin is not the dissociation of a tetramer into two dimers; instead, the key question is how to improve the solubility characteristics of the dimeric molecule without disturbing the ability of each subunit to fold into a functional dimeric form.

Expression and Purification of a Dimeric Streptavidin, Stv-43. A streptavidin mutant, Stv-43, which has the deletion of the G113–W120 loop in addition to the H127D mutation, was expressed efficiently in *E. coli* (Fig. 2*A*). Purification of expressed Stv-43 was attempted by the methods used for other streptavidin variants (26, 29, 30). However, no soluble dimeric molecule was obtained, suggesting that Stv-43 is unable to fold correctly or it has insufficient solubility under the conditions used. These possibilities were tested by modifying the purification procedure. The major modification was to include excess biotin in renaturation solutions. Biotin might act like a

molecular chaperone, allowing denatured, monomeric Stv-43 to fold correctly around it. Biotin binding would also improve solubility by sealing hydrophobic amino acid residues in the biotin-binding site and by providing an additional negative charge derived from the carboxyl group that is exposed to solvent.

The addition of biotin during renaturation generated a soluble dimeric streptavidin-like protein, judged from its molecular mass, which was detected by both SDS/PAGE (<13 kDa) and gel filtration chromatography (23 kDa; data not shown). To determine if this 23-kDa protein is Stv-43, excess D-[*carbonyl*-^{14}C]biotin was added to the crude 23-kDa protein fraction and allowed to exchange with bound biotin by incubation at room temperature for 1 hr. The resulting mixture was analyzed by gel filtration chromatography, monitoring the radioactivity derived from D-[*carbonyl*-^{14}C]biotin by liquid scintillation counting. Radioactivity was detected with the 23-kDa protein, in addition to free, unbound D-[*carbonyl*-^{14}C]biotin (data not shown). This demonstrates that the 23-kDa protein is able to bind biotin, and thus it is a functional dimeric streptavidin. These results also indicate that the presence of biotin during renaturation successfully allowed denatured, monomeric Stv-43 to fold correctly into a functional dimeric form.

To remove biotin bound to Stv-43 for further purification, crude Stv-43 was subjected to three sequential dialysis steps. The first dialysis is against TTBS to remove unbound biotin; the second is against TTBS containing urea to remove bound biotin; and the third is against TTBS to remove urea. In the second dialysis step, urea is used not as a denaturant but as a

Table 2.   Subunit–subunit interaction free energies through the monomer–monomer interface in natural tetrameric streptavidin and its dimeric mutants

Free energy term	Natural streptavidin	Stv-33	Stv-43
Electrostatic (E_{el})	−22.3	−21.8	−12.8
Desolvation (ΔG_d)	−66.9	−66.9	−55.1
Entropic ($-T\Delta S_{sc}$)	50.4	50.4	42.3
Others (ΔG_{const})	11.0	11.0	11.0
Total	−27.8	−27.3	−14.6

All values are expressed as kilocalories per mole.

biotin analog that has a very weak affinity for avidin ($K_a = 28$ M^{-1}) (35, 36). A very low concentration of urea (10 mM) was used to allow bound biotin to exchange with urea without disturbing the structure of Stv-43. After the three sequential dialysis steps, the resulting protein solution was subjected to biotin-affinity chromatography, in which almost 100% of Stv-43 was bound to immobilized biotin. This indicates that these dialysis steps efficiently dissociated bound biotin from Stv-43 without denaturing the protein. Stv-43 was eluted from immobilized biotin using a solution containing urea and biotin. Then, eluted proteins were dialyzed against TTBS containing biotin to remove urea. This fraction showed a single band at approximately 13 kDa by SDS/PAGE (Fig. 2*B*) and has a single peak at 23 kDa by gel filtration chromatography (Fig. 3*A*), indicating that Stv-43 was purified to homogeneity as a biotin complex.

Purified Stv-43 in the presence of excess biotin was stable at 4°C. However, without bound biotin, Stv-43 gradually dissociated into monomeric forms. Gel filtration chromatography (Fig. 3*B*) shows that only the remaining dimeric Stv-43 bound biotin, whereas no biotin binding was detected with the monomeric forms. This result suggests that the monomeric molecules cannot maintain a functional three-dimensional structure. Free energy calculations on the subunit interaction at the monomer–monomer interface of Stv-43 (Table 2) show that, in the absence of biotin, its E_{el} should be significantly higher (-12.8 kcal/mol) than that of the equivalent subunit pair in natural streptavidin (-22.3 kcal/mol). This suggests that the monomer–monomer interaction of Stv-43 may be weakened by the mutations made, resulting in insufficient stability to maintain a dimeric structure without biotin. Once the dissociation of subunits occurs, the resulting monomeric molecule is likely to denature because its β-barrel structure would not be maintained stably without the interaction with the adjacent subunit which has a complementary curvature at the monomer–monomer interface and makes numerous inter-subunit van der Waals interactions.

Biotin-Binding Characteristics of Stv-43. Purified Stv-43 without biotin bound greater than 0.95 molecules of biotin per subunit, as does natural streptavidin. However, the biotin-binding affinity constant (K_a) of Stv-43 was reduced to 1.5×10^7 M^{-1} at 4°C at pH 7.4. This is approximately one order of magnitude smaller than that of a tetrameric streptavidin mutant, Stv-38, in which a W120F mutation was made to weaken the intersubunit contacts made by W120 to biotin through the dimer–dimer interface (18). This suggests that F120 in Stv-38 makes weak intersubunit contacts to biotin through the dimer–dimer interface. The biotin-binding affinity constant of Stv-43 is in good agreement with that of a tetrameric streptavidin mutant, in which a W120A mutation was made to disrupt almost completely intersubunit contacts made by W120 to biotin (8.6×10^6 M^{-1}) (19). [Note that the conditions used for the determination of the biotin-binding affinity by Chilkoti *et al.* (19) are different from those in this study.] These comparisons reveal that intersubunit contacts made by W120 to biotin bound by an adjacent subunit make a considerable contribution to extremely tight biotin binding by tetrameric streptavidin. They also demonstrate the importance of subunit association and intersubunit communications in the biotin-binding characteristics and structural stability of this multisubunit protein.

Conclusions. Dimeric streptavidins have been designed by molecular modeling using enhanced binding free energy calculations, and one such dimeric molecule has successfully been produced in a functional form that is capable of binding biotin. Although this dimeric streptavidin, Stv-43, requires biotin to fold into a functional dimeric form and has limited stability

without bound biotin, success of the production of a functional dimeric molecule demonstrates the feasibility of designing dimeric streptavidins that have folding, stability, and biotin-binding affinity equivalent to natural streptavidin.

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