Engineering a novel, stable dimeric streptavidin with lower isoelectric point

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Abstract

We have engineered a soluble, stable two-chain dimeric streptavidin (TCD) in Escherchia coli. Examination of the three-dimensional structure of streptavidin aided by empirical binding free-energy calculations helped us to select mutations at subunit interfaces that dissociate the native tetramer and stabilize the desired dimer. We chose positions W120, L124, V125 and H127 and mutated them to 120D/124D/125D/127D (TCD-1); 120D/124N/125S/127D (TCD-2); and 120D/124D/125S/127D (TCD-3). The H127D mutation creates electrostatic repulsion that disrupts the dimer–dimer interface, but leaves it very hydrophobic. Therefore, W120, L124 and V125 were mutated to hydrophilic residues to increase dimer solubility. Among the three candidates, TCD-2 gave the best result: a stable, active dimer with $K_d$ for biotin of $\sim 1 \times 10^{-7}$ M after purification by gel-filtration chromatography. The experimental results confirm the possibility of rational engineering of low-pI dimeric streptavidins. Reduced-size streptavidin mutants with a net negative charge may be more suitable than antibodies or wild-type streptavidin for the targeting step in radioimmunotherapy because they should clear faster from the bloodstream and the kidney.

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Keywords: Biotin binding; Dimeric streptavidin; Molecular modeling; Protein engineering; Protein–protein interaction; Radioimmunotherapy

Abbreviations: FPLC, fast-protein liquid chromatography; TCD, two-chain dimer; SCD, single-chain dimer

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1. Introduction

Because of their extraordinary affinity \( (K_d \leq 10^{-14} \text{ M}) \) for biotin, streptavidin and avidin have found widespread use in biotechnology. Their tetrameric \((\alpha_2)2\) quaternary structure, irreversible binding to biotin, and large size \((M_r \sim 60 \text{ kDa})\), however, limit their usefulness for some applications (phage and chip display, affinity purifications, pre-targeted radioimmunotherapy, etc.). Due to its low dissociation constant for biotin, streptavidin has been widely used for localization, as a capturing molecule for \textit{in vitro} detection and immobilization of biotinylated biomolecules, as well as for \textit{in vivo} imaging, targeting and drug delivery. Radiolabeled streptavidin serves as an efficient drug carrier in the targeting step of radioimmunotherapy where the tumors are pre-targeted with biotinylated antibodies rather than radiolabeled antibodies (Boerman et al., 2003; Gruaz-Guyon et al., 2005; Goldenberg et al., 2006). Although streptavidin behaves better than antibodies as the radioisotope carrier, its size is still much larger than ideal. In addition, it has a remarkably high accumulation rate in the kidney (Schechter et al., 1995). The kidney retains positively charged molecules longer than those with negative charge (Onda et al., 2001; Rennen et al., 2001). Therefore, in order to engineer a reduced-size streptavidin with potential uses in radioimmunotargeting (among other applications), we chose to replace hydrophobic amino acids at the dimer–dimer interface of streptavidin with negatively charged amino acid residues thereby both favoring an \( \alpha_2 \) quaternary structure and lowering the pI.

Efforts to engineer reduced-size proteins (dimers and monomers) to extend the applications of streptavidin have been underway for about a decade. For radioimmunotherapy, such reduced-size streptavidins should speed up the passage of radioactivity to the tumor targets while clearing from the kidneys at a faster rate. To be active in biotin-binding in patients, however, the dimers must remain soluble in water and not dissociate further. In the work described here we focused exclusively on making a dimeric streptavidin. Monomeric streptavidin (or avidin) derivatives have recently been produced in other laboratories (Qureshi et al., 2001; Qureshi and Wong, 2002; Laitinen et al., 2003; Wu and Wong, 2005) but none of these have yet been shown to be useful in radioimmunotherapy. The smaller size and the lower pI (leading to increased net negative charge) of a dimeric streptavidin should allow this protein to serve as an improved \textit{in vitro} diagnostic or therapeutic agent by reducing the disadvantageous pharmacokinetics of natural tetrameric streptavidin. In addition to such applications, streptavidin has been used for affinity purification of biotinylated proteins. However, its low dissociation constant prevents the recovery of the proteins back from the affinity matrix unless very harsh conditions are used. Therefore, engineering streptavidin mutants with lower biotin-binding affinities as well as reduced size should help to enlarge the repertoire of streptavidin/avidin technology. A recent paper describing a system for pretargeting with a streptavidin-antibody conjugate prior to radiolabeling with a bis-biotin reagent also provides an example of the utility of modifying the streptavidin component to lower its biotin-binding affinity (Hamblett et al., 2005).

Thus, there have been several reports of successful production of non-covalent dimeric \((\alpha_2)\) streptavidins (Sano et al., 1997b; Laitinen et al., 1999) and avidins (Laitinen et al., 1999) as well as covalently coupled, single-chain dimers (SCDs) (Nordlund et al., 2004; Aslan et al., 2005). Surprisingly, the SCD mutants we produced by phage display (Aslan et al., 2005) bound biotin-4-fluorescein with a 100,000-fold higher affinity than they bound biotin. This unexpected result provides a reversible dual-ligand system with the mutant streptavidin mediating between biotin and biotin-4-fluorescein (Doerr, 2005). However, none of the non-covalent two-chain dimers described thus far work well enough for practical applications. Therefore, we sought a new design to engineer an active and stable dimeric streptavidin.

Streptavidin \([\alpha_2\alpha_2]\) has two different subunit interfaces: a strong monomer–monomer interface between subunits in the \(\alpha_2\) dimer and a weaker dimer–dimer interface in the \(\alpha_2\alpha_2\) tetramer. Because of the weaker interactions, we chose to disrupt the dimer–dimer interface to form a two-chain dimer. We used simple free-energy calculations to guide selection of mutations (Aslan, 2005). The design had to address several considerations that are generally important when reducing the size of multi-subunit proteins (Sano et al., 1997b). Dissociation must be induced by a minimum number of mutations, and in streptavidin, replacement
Fig. 1. Key wild-type residues at the dimer–dimer interface that were mutated to convert tetrameric streptavidin into a two-chain dimer. A single primary dimer \((\alpha_2)\) is shown with atoms belonging to chains A and B colored gray and tan, respectively, except for the mutated residues which are shown in green (chain A) or blue (chain B). Red atoms identify the two bound biotins. (A) Dimer–dimer interface viewed along the two-fold symmetry axis (perpendicular to the page). Mutated residues in chain A are (from top to bottom) W120, L124, V125, and H127 (L124 and V125 overlap). The same residues appear for chain B in the symmetry-related positions (from bottom to top). (B) Oblique view of the dimer–dimer interface. Atoms are colored as in (A). Images were constructed using PyMOL (http://pymol.sourceforge.net/) based on PDB-1SWE.

of histidine-127 with an aspartate (H127D) suffices. Dissociation, however, also exposes to solvent the side chains of many hydrophobic amino acid residues located at the dimer–dimer interface. Thus, engineering a dimeric streptavidin with sufficient water solubility requires introducing mutations to reduce the hydrophobicity of that interface (Fig. 1). Finally, the proposed mutations should not cause the dimer to dissociate into monomers.

In this paper, we describe three constructs for the production of dimeric streptavidin: TCD-1, TCD-2 and TCD-3 (Table 1). TCD-2 displayed the best solubility and binding behavior, therefore, we characterized it in detail.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Amino acid sequence changes used to create soluble TCDs</th>
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<tbody>
<tr>
<td>First construct</td>
<td>Second construct</td>
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<tr>
<td>TCD-1</td>
<td>TCD-2</td>
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<tr>
<td>W120D</td>
<td>W120D</td>
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<tr>
<td>L124D</td>
<td>L124N</td>
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<td>V125D</td>
<td>V125S</td>
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<td>H127D</td>
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2. Materials and methods

Oligonucleotides were from Operon Technologies Inc. (Alameda, CA). PfuTurbo DNA polymerase was from Stratagene (La Jolla, CA); restriction enzymes and ligases were from New England Biolabs (Beverly, MA); and chemical reagents were from Sigma (St. Louis, MO).

2.1. Free-energy calculations

Coordinates of wild-type tetrameric streptavidin were obtained from the Protein Data Bank (1STP). Models of streptavidin variants were built with QUANTA (Accelrys, San Diego, CA) and steric clashes were removed by subjecting the complex to energy minimization using the CHARMM potential.

The electrostatic interaction energy was calculated by the Coulombic expression using the molecular mechanics potential function CHARMM 19 (Brooks et al., 1983) with the distance-dependent dielectric permittivity of \(\varepsilon = 4r\), where \(r\) is equal to distance. The desolvation energy was estimated by the atomic solvation parameter model (Eisenberg and McLachlan,
1986) as a linear combination of the accessible areas of different atoms using an updated set of solvation parameters (Vajda et al., 1994). 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 (Vajda et al., 1995).

2.2. Construction of expression vectors

The starting material for the expression vectors for the TCDs was pTSA-13, which was derived from pET-3a (Rosenberg et al., 1987) (4.6 kb) by inserting the 360 bp gene that encodes the minimum-sized, wild-type core streptavidin consisting of amino acid residues gly-16 to val-133. PCR-based, site-directed mutagenesis was used to introduce the desired changes (creating mutants TCD-1, TCD-2 and TCD-3, Table 1) with the oligonucleotide pairs shown in Table 2. Amplified linear plasmid DNAs containing the mutations were purified by 0.8% agarose gel electrophoresis and recircularized by blunt-end ligation with T4 DNA ligase at 16 °C overnight and transformed into Escherchia coli STBL2 competent cells (Invitrogen, Carlsbad, CA). Colonies selected by NdeI digestion (4.96 kb linearized DNA) were further confirmed by DNA sequencing. Nucleotide sequences for the TCD proteins TCD-1, TCD-2 and TCD-3 have been deposited in GenBank under accession nos. AY973587, AY973588, AY973589, respectively.

2.3. Expression

E. coli strains BL21(DE3)(pLysE) and BL21(DE3) (pLysS) carrying pTSA-13 as an expression vector encoding TCD proteins under T7 promoter control were grown overnight at 37 °C with vigorous shaking in 3 mL of LB supplemented with 150 μg/mL ampicillin and 25 μg/mL chloramphenicol. Cultures were diluted 1:100 with fresh LB, supplemented with the antibiotics as before, and incubated with shaking at 300 rpm at 37 °C. When the absorbance of the culture reached 1.0 for cells carrying pLysS and 0.6 for those carrying pLysE, 100 mM IPTG was added to a final concentration of 0.4 mM to induce the expression of the T7 RNA polymerase gene (under control of the lacUV5 promoter). Cells were collected by centrifugation after incubating at 37 °C for 5 h with shaking.

2.4. Isolation and folding

We tried three different protocols for the isolation and folding of TCDs. Their distinguishing features were: (1) slow dialysis of protein in 7 M Gu–HCl against pH 4 buffer; (2) dropwise addition of protein in 7 M Gu–HCl to pH 6 buffer; and (3) same as protocol (2), but omitting the DNaseI and RNase digestion step during isolation.

Table 2
Primer name | Primer sequence | Tm
---|---|---
TCD-1-F | 5'-pCACGGACGACGGCAGACACCTTACCACAGGTGTAAGCGTCCG-3' | 81 °C
TCD-1-R | 120 | 125 | 127
TCD-2-F | 5'-pCACGAATTCCCGGACGACACCTTACCACAGGTGTAAGCGTCCG-3' | 80 °C
TCD-2-R | 120 | 125 | 127
TCD-3-F | 5'-pCACGGACTCGGACGACACCTTACCACAGGTGTAAGCGTCCG-3' | 81 °C
TCD-3-R | 120 | 125 | 127

Changes from the original sequence are shown in bold and underlined. Numbers correspond to mutated amino acid residues (Table 1). Stop codon TAG is shown in bold and vector sequences are given in italics. F and R refer to forward and reverse primer, respectively. Note that all the primers have 5'-phosphorylated ends.
In the first protocol (Aslan, 2005), adapted from (Sano et al., 1997a), cells were washed with 10 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA then resuspended in 30 mM Tris–HCl, pH 8, 0.1% Triton X-100, 2 mM EDTA (buffer A) and lysed by a freeze-thaw cycle three times, with vortexing between cycles. Following two 30 s-rounds of sonication with a Branson Sonifier cell disruptor on power setting four to reduce viscosity, 12 mM MgSO4, 10 μg/mL DNaseI, and 10 μg/mL RNase were added and the suspension was incubated for 30 min at room temperature, then centrifuged at 18,000 × g for 20 min. The insoluble fraction (which has the expressed streptavidin) was resuspended and washed once in 50 mM Tris–HCl, pH 8, 10 mM EDTA, 1.5 mM NaCl, 1 mM PMSF, 0.5% Triton X-100 and four times in the same buffer without Triton X-100. Then the inclusion bodies were dissolved in a volume of 7 M Gu–HCl (pH 1.5) equal to the volume of the induced culture. The solution was dialyzed very slowly against 0.2 M NH4OAc (pH 4), 10 mM EDTA, 0.02% NaN3, 0.02% Tween-20, since fast renaturation of chimeric streptavidins can cause them to aggregate, reducing the yield. Finally the protein was dialyzed against water, frozen in dry ice/ethanol and then lyophilized. Lyophilized TCD-2 samples were dissolved in buffers sterilized by passage through a 0.22 μm filter (Millipore). Buffers covered the range of pH 4–11 at unit intervals. The activity of TCD-2 was tested with 3H-biotin (Sano et al., 1997b; Aslan, 2005).

The second protocol followed the same procedure as the first up through the preparation of the inclusion bodies. After the washing step, inclusion bodies were first dispersed in deionized water (0.2–1 mL of H2O/6 liters of culture) to form a milky suspension to which 7 M Gu–HCl (pH 1.5) was added, followed by vortexing till no insoluble particles remained. The suspension was allowed to equilibrate for several hours at 4 °C, then centrifuged at 18,000 × g for 10 min at 4 °C to remove any precipitate. Solubilized protein was then diluted dropwise (one drop per 5 s) from a syringe (27 gauge, 1.5 in.) into folding buffer (50 mM MES, pH 6, 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF) at 4 °C while stirring forcefully. Folding buffer volume was half that of the induced culture. After equilibration overnight, the resulting solution was centrifuged to remove insoluble material and concentrated in Millipore Centriprep and Centricon centrifugal filter devices. This folding procedure is an adaptation (Aslan et al., 2005) of the method of Chu et al. (1998).

The third protocol followed the same procedure as the first up to the preparation of the cell lysate (which was in buffer A). Cells were first lysed by four freeze-thaw cycles with vortexing between each and then sonicated five times. Between each round (three cycles of 30 s on power setting 4, followed by 30 s off), samples were centrifuged at 18,000 × g for 10 min and resuspended in buffer. Sonication was repeated twice in buffer A and three times in 50 mM Tris–HCl, pH 8, 0.5% Triton X-100, 1 mM EDTA, 100 mM NaCl, 0.1% NaN3 (buffer B). After the last round, pellets were resuspended in buffer B without Triton X-100, then centrifuged at 18,000 × g for 10 min, resuspended in deionized water and folded by dilution into folding buffer as described in the second protocol (see above).

2.5. Purification/analysis by FPLC

We used high-performance gel filtration columns (Superdex 75, Amersham Pharmacia Biosciences) with an LCC–501 Plus system. Crude TCD-2 (ca. 100 μg, determined by 15% SDS-PAGE) was run on the column to determine its quaternary structure. The column was calibrated by using streptavidin, chymotrypsinogen A and ribonuclease A. The flow rate was 0.5 mL/min; the running buffer was 50 mM NH4OAc, pH 4, 150 mM NaCl.

2.6. Batch-style ion-exchange purification with DEAE Sepharose FF

We first attempted to purify TCD-2 protein by DEAE Sepharose FF column chromatography (column was equilibrated and washed with 20 mM Tris–HCl, pH 8.5, and protein was eluted with increasing salt concentration (50, 150, 300, 450 mM) in the same buffer). However, the protein did not bind to the column, but came off in the wash fraction. Therefore, we used batch-style ion-exchange purification instead. A 200 μL portion of DEAE Sepharose FF anion-exchange matrix (Amersham) was equilibrated at room temperature with 10 volumes of 20 mM Tris–HCl, pH 8.5. Then the mixture was centrifuged and decanted. Protein solution in the same buffer (30 μL, ca. 2 μg/μL) was mixed with beads for
1–2 min. After centrifugation for 5 min at 2000 × g a 5 μL aliquot of the supernatant was tested for protein using the Coomassie™ Plus-200 Protein Assay Reagent (Pierce, Woburn, MA). A blue color indicated the presence of the protein; the control reaction with 5 μL of buffer gave no color. Then the remaining supernatant (ca. 40 μL), separated from the beads, was dried completely by speed-vac treatment before dissolving in 10 μL of water. To this sample 10 μL of 2X SDS-PAGE gel-loading buffer was added prior to running on 15% SDS-PAGE. The biotin-binding ability of the purified protein was tested by Ultrafree-MC centrifugal filtration units according to Sano et al. (1997b).

2.7. Biotin binding

We used two methods to examine biotin-binding to TCD proteins: (a) Sephadex G-25 gel filtration on PD10 columns (Amersham); and (b) centrifugal separation assays with Ultrafree-MC filtration units (Millipore, Bedford, MA) (Sano et al., 1997b). In both cases experiments were carried out at 25 °C and biotin concentrations were determined using d-[8,9-3H]biotin (28 Ci/mmol initial sp. act., Amersham) as a radiolabel, monitored by scintillation counting. The concentration of the purified protein was determined by A280 measurement using an extinction coefficient of 36,130 M⁻¹ cm⁻¹ as calculated using ProtParam (http://ca.expasy.org/cgi-bin/protparam).

2.8. Thermal stability

FPLC-purified protein was tested by 15% SDS-PAGE gel electrophoresis with and without boiling, wild-type tetrameric streptavidin served as a control.

3. Results

3.1. Molecular modeling and free-energy calculations

Two-chain dimeric streptavidin mutants were constructed by disrupting the subunit association at the dimer–dimer interface (Fig. 1). We devised three sets of candidate dimer–dimer interface changes for site-directed mutagenesis of streptavidin at four positions to create stable, soluble dimers with retention of high biotin affinity (Table 1). The selection of these candidates was based on the examination of the three-dimensional structure of streptavidin complemented by simple hydrophobicity and electrostatics calculations (see Section 2).

Position 127 was mutated to aspartate to create an electrostatic interaction that prevents the association of dimers to form a tetramer. Calculations indicate that tryptophan-120 is the most hydrophobic side chain exposed to solvent in the proposed dimeric molecule; hence, we selected the W120D mutation to improve the free energy of solvation for all three candidates. Both glutamate and aspartate were considered as potential replacements for tryptophan, but because of its higher hydrophilicity, we chose to use aspartate. For the remaining two positions (124 and 125) we selected aspartate residues in the case of TCD-1, thereby maximizing polarity and negative charge. In the case of TCD-2, neutral polar amino acids were chosen (asparagine at position 124 and serine at position 125) to increase desolvation free energy without contributing additional negative charges. Finally, for TCD-3 we selected aspartate at position 124 and serine at position 125 to have an intermediate case.

The calculations for TCD-2 supported the possibility of a soluble dimeric mutant (see Section 4). Among all three candidates, we thought that it would give the most soluble, stable dimer since the charge changes the least (overall change in charge at neutral pH is −2), whereas the overall changes in charge at neutral pH for TCD-1 and TCD-3 are −4 and −3, respectively, resulting in less favorable electrostatic interactions through the monomer–monomer interface. The experimental results supported this expectation, and although all three constructs were successfully overexpressed, we characterized only TCD-2 in detail.

3.2. Expression

Each dimeric streptavidin mutant was expressed efficiently in E. coli as monitored by 15% SDS-PAGE and gave the expected monomer band. Fig. 2 shows representative expression of TCD-2. Similar results were obtained for TCD-1 and TCD-3.

3.3. Folding and purification of TCD-2

The expressed streptavidins formed insoluble aggregates in the cells. However, dialysis of these inclusion
Fig. 2. Expression of TCD-2 protein in E. coli carrying pTSA-13 monitored on 15% SDS-PAGE. Lane headings: M, protein marker (in kDa indicated at left), U, uninduced culture, I, induced culture; 0, 4.5 h after start of induction. The thick, dark bands toward the bottom of the lanes marked 4.5-I correspond to TCD-2 (arrows). Expression was induced in 200 mL culture volumes at 37°C with 0.4 mM IPTG. Inclusion bodies from 100/L9262H of culture were loaded on the gel. The marker lane contained 14.4/L9262H protein. For each expression system the first lane shows the result of electrophoresis of uninduced cells at time zero after cells were grown to OD600 = 0.6 for BL21(DE3)pLysE and OD600 = 1 for BL21(DE3)pLysS, the second lane shows the result after 4.5 h of induction with 0.4 mM IPTG, and the third lane shows the result for an uninduced control culture after 4.5 h.

bodies solubilized in 7 M guanidine hydrochloride (pH 1.5) gave soluble, renatured protein in every case. Gel-filtration chromatography confirmed that these proteins are dimeric ($M_r = 25$ kDa), cf. Fig. 3. The minor peak at a retention volume of ca. fourteen milliliters in Fig. 3 is probably (inactive) monomer.

TCD-2 was folded at pHs 4, 5 and 6 by following the first protocol (cf. Section 2). Although the protein yield was the highest at pH 5 (100 mg/L culture), the binding activity was lower than that obtained at pH 4. The yield at pH 4 was about 50 mg/L culture. pH 6 gave the lowest protein yield (20 mg/L culture) and binding activity. PD-10 column experiments between pH 4–11 showed that TCD-2 has the highest biotin-binding activity at pH 4, though appreciable binding occurs at higher pH values including pH 7 (see Section 3.4).

Analyses of protein from the first isolation/folding protocol showed that TCD-2 is stable and active for at least 2 months at pH 4 or 5 at 4°C. This protein is about 80% pure after folding (as estimated from SDS-PAGE). Lyophilized protein stored at −80°C, then resuspended in the appropriate buffer retained its $^3$H-biotin binding activity for at least 3 months. However, TCD-2 stored at −80°C for 3 months in pH 11 buffer (50 mM Na2CO3 and 150 mM NaCl) lost activity.

The second isolation/folding protocol takes less time by avoiding dialysis of the denatured protein against a large volume of Gu–HCl (and also saves money since Gu–HCl is rather expensive). TCD-1, TCD-2 and TCD-3 produced from 100 mL E. coli cultures using the second method showed yields of ca. 300 μg/1 L culture, ca. 1 mg/1 L culture and ca. 150 μg/1 L culture, respectively, as estimated from 15% SDS-PAGE (data not shown). However, no $^3$H-biotin binding activity was observed for TCD-1 and only weak binding for TCD-3, as performed with Ultrafree-MC centrifugal filtration units (Sano et al., 1997b; Aslan, 2005; Aslan et al., 2005). Because of this, TCD-1 and TCD-3 were not characterized further.

The third protocol is a streamlined expression and purification of TCD proteins, which takes the least time to obtain purified protein (cf. Section 2). Thus, among the three expression protocols we recommend the last one. However, when these protocols were used for TCD-2 we obtained active protein that bound biotin in every case.

Folded TCD-2 protein was purified with a batch-style, ion-exchange adsorption purification with DEAE Sepharose FF. SDS-PAGE (15%) showed only one band at the position expected for the monomeric streptavidin (Fig. 4), and we estimated that it was
at least 90% pure based on inspection of the gel. The purified protein gave a positive $^3$H-biotin-binding result.

3.4. Biotin-binding ability/affinity

Gel-filtration experiments with Sephadex G-25 nicely demonstrated that TCD-2 bound biotin as a dimer (Figs. 5 and 6). These assays also clearly displayed pH-dependent binding of biotin to the protein by the presence of a fast-eluting peak that diminished in size with increasing pH, but separated at the lower pHs from the more slowly eluting free-biotin peak. Moreover, the best binding occurred at pH 4. Fig. 6 presents a very similar experiment to Fig. 5C. However, since the biotin concentration was reduced from 14 to 0.13 nM for the experiment shown in Fig. 6, the peak corresponding to the TCD-biotin complex is cleanly separated from the large peak containing free biotin.

The clear separation between bound and unbound $^3$H-biotin seen in Fig. 6 indicates tight binding at pH 4. From a plot of bound biotin versus free biotin, determined by measurements with Ultrafree-MC centrifugal filtration units, we estimated $K_d$ as $2.5 \times 10^{-7}$ M, the concentration of $^3$H-biotin at 50% saturation (Fig. 7). $K_d$ calculations were based on the assumption that there are two non-cooperative biotin binding sites per dimer since the binding curve was hyperbolic (Fig. 7).

3.5. Effects of pH and concentration

Experiments on TCD-2 with Ultrafree-MC centrifugal filtration units showed that the effects of pH change from 4 → 7 → 4 and 4 → 10 → 4 are reversible; the protein regained full activity after exposure to pH 7 or pH 10. However, it had essentially no binding activity at pH 10, though some was retained at pH 7 (data not shown, cf. Fig. 5). In addition, dilution up to 4.4-fold had no apparent effect on binding.

3.6. Thermal stability

Unboiled dimeric streptavidin behaves the same way on an SDS-PAGE gel electrophoresis as boiled samples, giving only a monomer band (Fig. 8). However, as previously reported (Reznik et al., 1996), while boiled tetrameric streptavidin migrates as a monomer, the unboiled protein remains tetrameric on SDS-PAGE gel electrophoresis (Fig. 8). Our experiment demonstrates that, as expected, the quaternary structure of the two-chain dimer is weaker than that of native streptavidin. Note that due to the mutations, the mobility of the TCD monomer is somewhat less than that of the wild-type monomer, and less than that expected for the calculated molecular weight based on the TCD amino acid sequence (12.5 kDa).
is no obvious explanation for this deviation, although even more extreme cases have been described [such as histone H1, a monomer that has a MW of 21 kDa but migrates at 30 kDa on SDS-PAGE (van Holde et al., 1998)].

Fig. 8. SDS-PAGE (15%) of boiled and unboiled two-chain dimeric streptavidin compared with wild-type tetrameric streptavidin. Lanes 1, 4 and 7: molecular weight markers (sizes in kDa listed at left). Lanes 2 and 3: TCD-2, boiled and unboiled, respectively. Lanes 5 and 6: wild-type tetrameric streptavidin, boiled and unboiled.

4. Discussion

The most important step in the design of our dimeric streptavidin is the H127D mutation. Since the newly introduced aspartate side chains have negative charges at pH > 4 they yield strong repulsive electrostatic interactions. Calculations show that the H127D mutation alone destabilizes the dimer–dimer interaction by a free energy increase of some 20 kcal/mol (Sano et al., 1997b). Thus, the tetramer (i.e., a dimer of the dimers) cannot form. However, the dimer–dimer interface remains extremely hydrophobic, with a calculated desolvation free energy of −64.2 kcal/mol, nearly twice that for typical complexes of small globular proteins (Vajda et al., 1994). Sano et al. (1997b) first tried to create a two-chain dimeric streptavidin by mutating the single residue (H127D), but this formed insoluble aggregates during folding—a result consistent with the calculations. Another streptavidin mutant, stv-43, generated by deletion of the G113-W120 loop in addition to the H127D substitution (Sano et al., 1997b) was also expressed efficiently in E. coli.; loop deletion made the calculated desolvation free energy less negative. However, this protein only folded stably in the presence of biotin—acting as a chaperone. In the absence of biotin, it gradually dissociated into monomers.

The goals of the improved design presented in this paper were (a) to further decrease tetramer stability by increasing the desolvation free energy and (b) to keep the monomer–monomer binding free energy close to
that of the original. Since neither the van der Waals nor the entropic contributions to the monomer–monomer binding free energy are significantly affected by the mutations, we focused on retaining the electrostatic interaction energy close to the $-22.3 \text{kcal/mol}$ as seen in the wild-type (Sano et al., 1997b). With a view to potential applications in radioimmunotherapy, we selected mutations that decreased the calculated isoelectric point from the original predicted value of 6.03 [http://ca.expasy.org/tools/pi_tool.html] since positively charged molecules have undesirable higher kidney retention. Calculated pIs are 4.51, 4.83 and 4.65 for TCD-1, TCD-2 and TCD-3, respectively.

Mutation of all four selected nonpolar positions to negatively charged residues brought the calculated desolvation free energy close to target. However, the electrostatic energy through the monomer–monomer interface was calculated to be some 4 kcal/mol higher than in the native dimer, an undesirable degree of destabilization. Hence we also chose to introduce polar rather than charged residues at one or two positions. The dimer–dimer desolvation and the monomer–monomer electrostatic term for TCD-2 best met the conditions we defined, predicting the existence of a soluble dimeric mutant, and this was confirmed experimentally as described. The other two mutants were expected to be less stable. The mutations were also expected to affect biotin binding. TCD-1 did not show any biotin-binding ability and TCD-3 showed weak binding. This is not surprising, since, compared with the wild-type streptavidin subunit, TCD-1 has four additional negative charges, two of which (D124 and D125) are close to the biotin binding pocket– and biotin is anionic. TCD-3, which has three additional negative charges per subunit, showed some binding, probably due to having a neutral S125 side chain instead of the negatively charged D125.

In general, storage of TCD-2 for a number of weeks at 4 $^\circ$C or months at $-20^\circ$C or $-80^\circ$C had little effect on the binding activity (as measured with $^3$H-biotin). Likewise neither pH-cycling nor dilution seemed to reduce its activity significantly (data not shown), suggesting that TCD-2 is reasonably robust. However, in contrast with native core streptavidin, TCD-2 dissociates in SDS without boiling (Fig. 8).

We estimated the $K_d$ of TCD-2 for biotin as $2.5 \times 10^{-7}$ M. In wild-type streptavidin the intersubunit contacts made by tryptophan-120 to the biotin bound by adjacent subunits contribute significantly to biotin binding (Hendrickson et al., 1989; Weber et al., 1989; Chilkoti et al., 1995; Sano and Cantor, 1995; Freitag et al., 1998); dimeric streptavidins which lack these contacts are expected to have reduced biotin affinity since mutation of W120 to phenylalanine or lysine decreased the biotin-binding affinity of tetrameric streptavidin by a factor of $10^7$ (Sano and Cantor, 1995; Laitinen et al., 1999) and mutation of W120 to alanine decreased it by a factor of $10^8$ (Chilkoti et al., 1995).

While our design appears to be reasonable and yields a stable dimer, another dimer results from a very different modification. This other design was inspired by the observation of a lysine residue at the position of sea urchin fibropellin (an avidin/streptavidin homolog) that corresponds to position 120 of streptavidin. The W120K mutant of streptavidin and the counterpart (W110K) mutant of avidin yielded stable dimeric proteins with $K_d$’s for biotin of about $10^{-8}$ M (Laitinen et al., 1999). However, it is important to note that the W120K mutant, while forming a dimer in dilute solution, crystallizes as a tetramer (Pazy et al., 2003). It also, of course, has a higher pI (calculated as 6.71) than that of wild-type streptavidin (6.03).

The results reported here could be employed to help design mutations to regulate the degree of association of the single-chain dimeric streptavidins we recently described (Aslan et al., 2005). These proteins “tetramerize” to form ca. 57 kDa oligomers via the same dimer–dimer interface that we have successfully manipulated in the work reported here. Furthermore, the basic single-chain dimer construct can also be further modified by directed-evolution techniques.

Our research on both single- and two-chain streptavidins has demonstrated ways to create reduced-size mutants with a range of biotin-binding affinities. The novel design of SCD mutants should prove useful for bacteriophage and array-display applications, while engineering two-chain dimeric streptavidins has helped us further to elucidate the subtle points of this macromolecule-ligand system. This study also contributes to general understanding of interactions stabilizing the quaternary structure of streptavidin that may apply to other systems as well, including components.
of some of the many variant radioimmunotherapy protocols currently under investigation (Gruaz-Guyon et al., 2005).

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References


