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Intersubunit contacts made by tryptophan 120 with biotin are essential for both strong biotin binding and biotin-induced tighter subunit association of streptavidin

(protein engineering/biotin-binding protein/avidin)

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ABSTRACT **In natural streptavidin, tryptophan 120 of each subunit makes contacts with the biotin bound by an adjacent subunit through the dimer–dimer interface. To understand quantitatively the role of tryptophan 120 and its intersubunit communication in the properties of streptavidin, a streptavidin mutant in which tryptophan 120 is converted to phenylalanine was produced and characterized. The streptavidin mutant forms a tetrameric molecule and binds one biotin per subunit, as does natural streptavidin, indicating that the mutation of tryptophan 120 to phenylalanine has no significant effect on the basic properties of streptavidin. However, its biotin-binding affinity was reduced substantially, to approximately 10^8 M^{-1}, indicating that the contact made by tryptophan 120 to biotin has a considerable contribution to the extremely tight biotin binding by streptavidin. The mutant retained bound biotin over a wide pH range or with the addition of urea up to 6 M at neutral pH. However, bound biotin was efficiently released by the addition of excess free biotin due, presumably, to exchange reactions. Electrophoretic analysis revealed that the intersubunit contact made by tryptophan 120 to biotin through the dimer–dimer interface is the major interaction responsible for the biotin-induced, tighter subunit association of streptavidin. In addition, the mutant has weaker subunit association than natural streptavidin even in the absence of biotin, indicating that tryptophan 120 also contributes to the subunit association of tetramers in the absence of biotin.**

Streptavidin, a protein produced by *Streptomyces avidinii*, binds *d*-biotin (vitamin H) with a remarkably high affinity (1–3). The extremely tight biotin-binding ability of streptavidin not only offers useful bioanalytical applications (4, 5) but also generates considerable protein chemical interest, particularly as an attractive model for studying macromolecule–ligand interactions (6–13). One such effort was made by determining the three-dimensional structure of streptavidin by x-ray crystallography (14, 15). This has set the stage for developing an understanding of the unusual properties of streptavidin.

One unusual structural feature of streptavidin shown in the crystal structures is the contacts made by tryptophan (Trp)-120 of one subunit with biotin bound by an adjacent subunit through the dimer–dimer interface (see Fig. 1*A*), where two stable symmetric dimers are associated to form a tetramer having dihedral D_2 symmetry (16). This tryptophan residue contacts the alkyl moiety of the pentanoyl group of biotin in an apparent hydrophobic interaction. This intersubunit contact of Trp-120 with biotin ought to contribute to the extremely tight biotin binding by streptavidin. We recently showed that the subunit association of streptavidin is considerably tightened upon biotin binding (17). Because the contact made by Trp-120 to biotin occurs through the dimer–dimer interface, this intersubunit communication is likely to play a key role in the biotin-induced tighter subunit association of streptavidin.

In the present work, a streptavidin mutant was designed in which Trp-120 was converted to phenylalanine (Phe), which should have a considerably reduced contact with biotin. The mutant was produced by genetic engineering and characterized to understand quantitatively the role of Trp-120 and its intersubunit communication in the properties of streptavidin.

MATERIALS AND METHODS

Materials. An oligonucleotide-directed *in vitro* mutagenesis kit and *d*-[*carbonyl*-^{14}C]biotin were obtained from Amersham. Natural core streptavidin was purchased from Boehringer Mannheim and was used without further purification; 2-iminobiotin-agarose was obtained from Sigma. An oligonucleotide was synthesized on an automated DNA synthesizer by using β-cyanoethyl phosphoramidite chemistry (Operon Technologies, Alameda, CA).

Construction of an Expression Vector. An expression vector was constructed by using pTSA-13 (T.S., M. W. Pandori, X. Chan, and C.R.C., unpublished data) as the starting material, which carries a truncated streptavidin gene encoding amino acid residues 16–133. mpSA-29 was first generated by inserting a 480-bp fragment of pTSA-13 carrying the entire coding sequence into M13mp18. Oligonucleotide-directed *in vitro* mutagenesis (18) was performed on mpSA-29 by using the 30-nt oligonucleotide, 5′-ACCAGCGTGGACTTGAAG-GCGTTGGCCTCG-3′, to convert the TGG codon for Trp-120 to TTC for Phe (mpSA-36). Then, a 405-bp fragment of mpSA-36 carrying the entire coding sequence was cloned into pET-3a (19, 20). The resulting expression vector, pTSA-38 (see Fig. 2), encodes a streptavidin mutant (Stv-38) in which Trp-120 is replaced by Phe.

Expression and Purification. Expression of Stv-38 was carried out by using the T7 expression system (19, 20) as described (21–23). Briefly, *Escherichia coli* lysogen BL21 (DE3) (19, 20) carrying pLysE (19) and pTSA-38 was grown at 37°C with shaking in LB medium (24) supplemented with 0.4% glucose, ampicillin at 150 μg/ml, and chloramphenicol at 25 μg/ml. When the OD_{600} 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 Stv-38 was performed by using BL21(DE3)(pLysE)(pTSA-38), which had been incubated for 4 h after induction, as the source. The purification procedure was the same as described (22, 23, 25), including 2-iminobiotin-affinity chromatography (26).

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Determination of Biotin-Binding Affinity. Biotin-binding affinity was determined by an equilibrium dialysis method by using a microdialyzer (Hoefer). Into two opposing 100-μl Teflon chambers separated by a dialysis membrane were placed 100 μl each of *d*-[*carbonyl*-^{14}C]biotin (2 nM–4 μM; 53 mCi/mmol; 1 Ci = 37 GBq) and Stv-38 (5.3 μg/ml; 0.42 μM subunits) solutions, both in Tris-buffered saline (TBS; 150 mM NaCl/20 mM Tris·HCl, pH 7.4/0.02% NaN_3), respectively. The chambers were incubated at 30°C with rotation until equilibration (≈48 h), and the concentration of *d*-[*carbonyl*-^{14}C]biotin in each chamber was determined by liquid scintillation counting. The results were analyzed by Scatchard plots (27), from which apparent biotin-binding affinity was estimated.

Determination of Biotin-Binding Stability. The biotin-binding stability of Stv-38 was investigated by varying the pH, by adding urea at neutral pH, or by adding free biotin at neutral pH. Purified Stv-38 was mixed with *d*-[*carbonyl*-^{14}C]biotin, both in TBS, at a molar ratio of biotin to biotin-binding site of 1.0 to saturate the biotin-binding sites. This Stv-38 solution was then mixed with an equal volume of an appropriate solution to adjust the final pH, urea concentration, or free biotin concentration. Varying the final pH of the solution was accomplished by using a concentrated wide-range buffer, consisting of 480 mM acetic acid, 480 mM boric acid, and 480 mM phosphoric acid. The pH was adjusted with NaOH. Urea and biotin solutions were prepared in TBS at concentrations up to 12 M and 660 μM, respectively. The mixture was incubated at 21°C for 20 min, transferred to an Ultrafree MC filter unit (Millipore), and centrifuged at 1600 × *g* for 10 min. The amount of released biotin was determined from the radioactivity of the filtrate. Natural core streptavidin was also analyzed as the control.

Effect of Biotin Binding on Subunit Association. The effect of biotin binding on subunit association of Stv-38 was investigated by an SDS/PAGE method (17). Stv-38 (4.0 μg; 0.32 nmol of subunits), in 4 μl of TBS, was mixed with an equal volume of TBS with or without 1.7 nmol of biotin (molar ratio of biotin to subunit, 5.3), and the mixtures were incubated at 21°C for 5 min. To each of these mixtures (8 μl), 2 μl of 3.0% (wt/vol) SDS/100 mM Tris·HCl, pH 6.8/40% (vol/vol) glycerol was added to a final SDS concentration of 0.6%. The resulting samples were either incubated at 21°C for 5 min or heated in boiling water for 3 min and then subjected to SDS/PAGE. Natural core streptavidin was used as the control.

Gel-Filtration Chromatography. Gel-filtration chromatography was carried out at 21°C on a Superdex 75 HR 10/30 column (1.0 × 30 cm; Pharmacia) by using a fast protein liquid chromatography (FPLC) system (Pharmacia). Detailed conditions are given in the legend to Fig. 4. The molecular mass of Stv-38 was estimated from the column by calibration with molecular mass protein standards (Boehringer Mannheim) and natural core streptavidin.

Biotin-Binding Ability. Biotin-binding ability was determined by a gel-filtration method (28) using *d*-[*carbonyl*-^{14}C]biotin and PD-10 columns (Pharmacia).

SDS/PAGE Analysis. Proteins were analyzed by SDS/PAGE through 15% polyacrylamide gels set up in a discontinuous buffer system (29). Proteins were stained with Coomassie brilliant blue R-250.

Determination of Protein Concentrations. The concentrations of Stv-38 and natural core streptavidin were determined from the absorbance at 280 nm by using extinction coefficients of 3.0 and 3.35 (17), respectively.

RESULTS AND DISCUSSION

Design of a Streptavidin Mutant with a Reduced Biotin-Binding Affinity. Although the direct participation of tryptophan residues of streptavidin has been indicated in biotin binding (3, 30–33), no studies have yet been performed to understand quantitatively the contribution of Trp-120 and its intersubunit communication to biotin binding by streptavidin. A primary reason for this is that, because streptavidin has six tryptophan residues per subunit (3, 34), targeted modifications of any one specific tryptophan residue are difficult by conventional chemical methods. The tetrameric nature of streptavidin makes targeted modifications even more difficult. These facts encouraged us to produce, by genetic engineering, a streptavidin mutant, which lacks the contact made by Trp-120 with biotin to understand the role of this residue and its characteristic intersubunit communication in the properties of streptavidin.

One key issue in designing such mutants is that Trp-120 may play an important role in maintaining local structures of streptavidin, particularly around the biotin-binding sites and the dimer–dimer interface. Very high hydrophobicity is observed around Trp-120, and three other tryptophan residues (Trp-79, -92, and -108) are clustered and make contacts with biotin (13–15, 33, 35) (see Fig. 1*A*). In addition, hydrophobic interactions are the major force for stable association of the two symmetric streptavidin dimers (S. Vajda, personal communication). Thus, drastic changes in local environment caused by the mutation of Trp-120 could prevent the molecule from correctly folding, resulting in the complete loss of biotin-binding ability. In fact, the conversion of some amino acid residues located around the dimer–dimer interface to hydrophilic amino acids caused the formation of insoluble aggregates, probably due to random intermolecular interactions (unpublished data). To effectively disrupt the intersubunit hydrophobic contact made by Trp-120 with biotin, without disturbing local environments around this residue, we decided

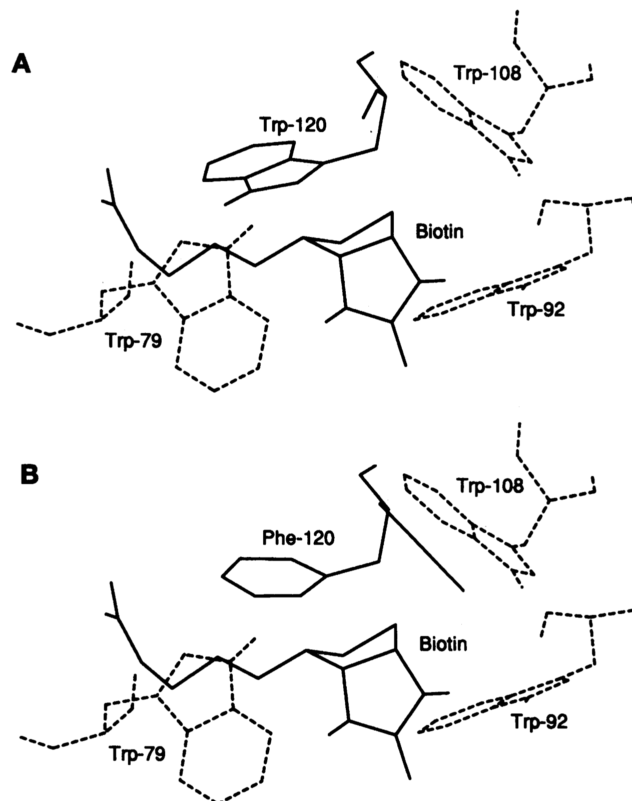

FIG. 1. Local structures around the biotin-binding site of streptavidin. (*A*) Natural streptavidin. (*B*) Stv-38, in which Trp-120 is converted to Phe. The positions of the four Trp residues (Trp-79, -92, -108, and -120) and biotin are shown. These structures are drawn on the basis of the known three-dimensional structure of natural streptavidin (14, 15). Note that Trp-79, -92, and -108 are from one streptavidin subunit with biotin, while Trp-120 or Phe-120 is provided by an adjacent subunit through the dimer–dimer interface.

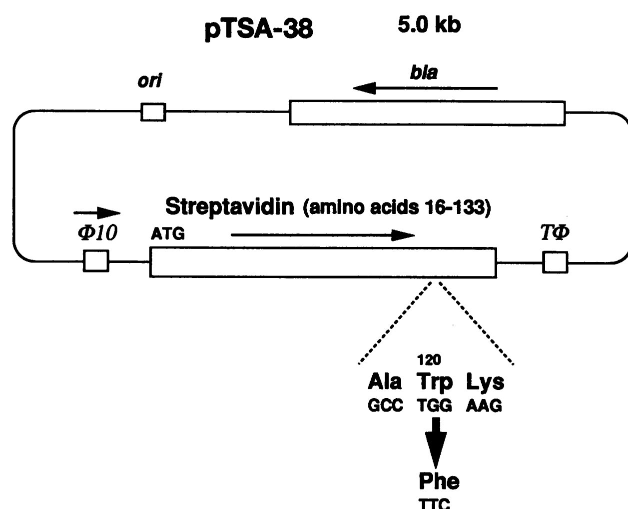

FIG. 2. Expression vector pTSA-38 for a streptavidin mutant with a reduced biotin-binding affinity (Stv-38). pTSA-38 carries the coding sequence for amino acids 16–133 of mature streptavidin (34), in which the codon for Trp-120 (TGG) has been converted to that for Phe (TTC). The coding sequence is placed under the T7 promoter (*Φ10*), followed by the transcription terminator of bacteriophage T7 (*TΦ*) (19, 20). *ori*, Origin of replication; *bla*, β-lactamase gene.

to convert Trp-120 to Phe. Because of its smaller size, the Phe residue, by displacing Trp-120 of one subunit, should have a considerably reduced hydrophobic interaction with the alkyl moiety of the pentanoyl group of biotin bound by an adjacent subunit (Fig. 1*B*). If no local conformational changes occurred as a result of this mutation, the minimal distance between the phenyl group of this Phe residue and the alkyl chain of biotin would be approximately 5.1 Å. This distance is significantly greater than that between Trp-120 and biotin in natural streptavidin (4.1 Å). However, because of its hydrophobicity, the conversion of Trp-120 to Phe would have minimal effects on the local hydrophobic environments around the biotin-binding sites and the dimer–dimer interface.

Expression and Purification of Stv-38. A standard oligonucleotide-directed mutagenesis approach was used to introduce mutations into the coding sequence of streptavidin. The expression vector pTSA-38 (Fig. 2) encodes a core streptavidin (16, 36) (amino acid residues 16–133), in which the codon for Trp-120 (TGG) has been changed to one for Phe (TTC). The T7 expression system (19, 20), which allows the efficient production of various recombinant streptavidins (21–23) and streptavidin-containing chimeras (25, 37), was used successfully to produce the encoded streptavidin mutant (Stv-38) in *E. coli* (Fig. 3*A*). Expressed Stv-38 was purified to homogeneity (Fig. 3*B*) by the method used for other recombinant streptavidins (22, 23, 25), which includes 2-iminobiotin-affinity chromatography (26). By SDS/PAGE, the subunit molecular mass of Stv-38 was estimated to be approximately 13 kDa, which is consistent with the molecular mass obtained from the deduced amino acid sequence (12.6 kDa).

Purified Stv-38 binds greater than 0.97 molecule of biotin per subunit, indicating that the molecule has full biotin-binding ability. By gel-filtration chromatography using a Superdex 75 HR 10/30 column (Fig. 4), the molecular mass of Stv-38 was estimated to be 49 kDa, indicating that Stv-38 forms a tetrameric molecule, as does natural streptavidin. These results demonstrate that the conversion of Trp-120 to Phe has no significant effect on the basic properties of streptavidin. They also reveal that this mutation has minimal effects on local environments around the biotin-binding sites and the dimer–dimer interface, thus allowing the correct folding of the molecule.

Biotin-Binding Affinity of Stv-38. Although no significant changes were observed in the basic properties of streptavidin by the mutation of Trp-120 to Phe, Stv-38 is very likely to have a reduced biotin-binding affinity because of the lack of the hydrophobic contact made by Trp-120 with biotin observed in natural streptavidin. To quantitatively determine the contribution of this intersubunit contact to the binding of biotin by streptavidin, the binding affinity of Stv-38 for biotin was investigated.

By equilibrium dialysis analysis, the biotin-binding affinity of Stv-38 was estimated to be 1–3 × 10^8 M^{-1} at pH 7.4 and 30°C. The biotin-binding affinities of a recombinant core streptavidin (T.S., M. W. Pandori, X. Chan, and C.R.C., unpublished data) and natural streptavidin are too high to be determined by the equilibrium dialysis used for Stv-38. Thus, the affinity of natural core streptavidin for biotin (4 × 10^{14} M^{-1}; ref. 3), estimated from the rate constants for binding and dissociation under slightly different conditions (pH 7.0 and 25°C), was used for comparison. This indicates that a substantial reduction in the biotin-binding affinity was caused by the mutation of Trp-120 to Phe and suggests that the hydrophobic contact made by Trp-120 with biotin has a considerable contribution to the extremely tight biotin binding by streptavidin. However,

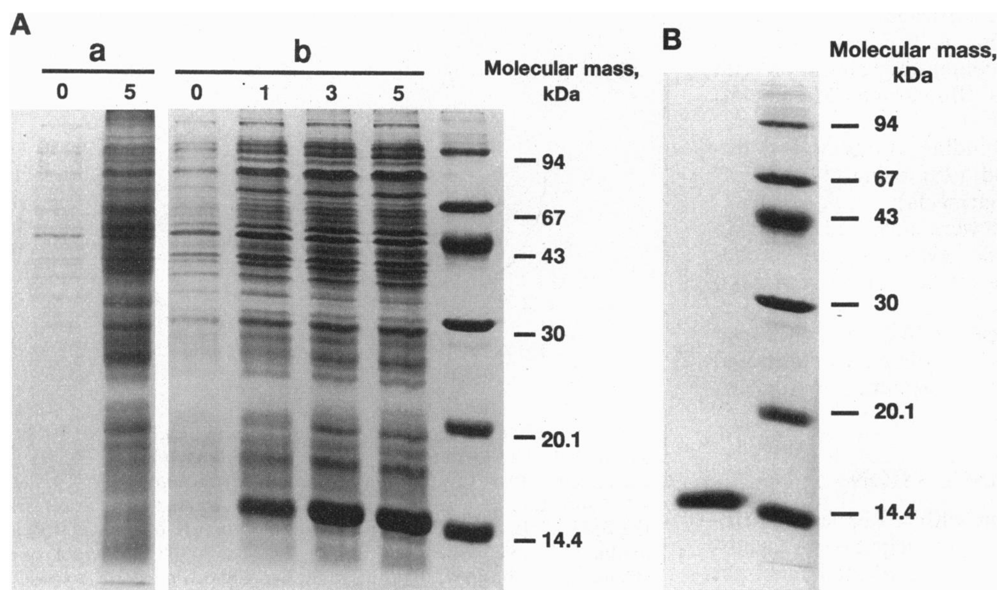

FIG. 3. (*A*) Expression of Stv-38 in *E. coli* by using expression vector pTSA-38. Total cell protein from BL21(DE3)(pLysE), with or without pTSA-38, was analyzed by SDS/15% PAGE. Lanes a, BL21 (DE3)(pLysE); b, BL21(DE3) (pLysE)(pTSA-38). The number above each lane is the time in hours after induction. Each lane contains the total cell protein from the following volume of culture: At 0 h for a and at 0 h and 1 h for b, 50 μl; at 3 h and 5 h for b, 33 μl; and at 5 h for a, 25 μl. The right lane contains molecular mass protein standards. (*B*) SDS/PAGE analysis of purified Stv-38. Approximately 3 μg of purified Stv-38 was applied to an SDS/15% polyacrylamide gel. The right lane contains molecular mass protein standards.

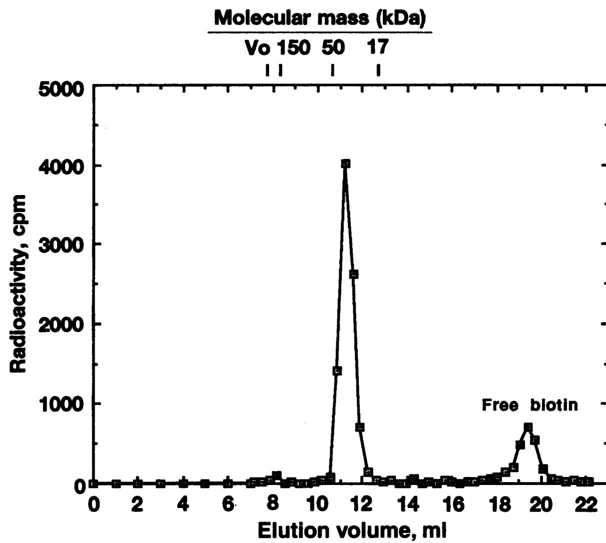

FIG. 4. Gel-filtration chromatography of Stv-38. Purified Stv-38 (≈220 ng) was saturated with biotin by the addition of excess *d*-[*carbonyl*-^{14}C]biotin. The mixture was applied to a Superdex 75 HR 10/30 column (1.0 × 30 cm; Pharmacia) previously equilibrated with TBS. Proteins were eluted at 21°C with TBS at a flow rate of 400 μl/min, and 340-μl fractions were collected. The radioactivity of each fraction was quantitated by liquid scintillation. The molecular mass of Stv-38 was estimated by calibration with molecular mass protein standards and natural core streptavidin (Boehringer Mannheim). The positions where the molecular mass standards eluted are shown at the top. The protein standards were sheep IgG, 150 kDa; Fab fragment of sheep IgG, 50 kDa; and horse skeletal muscle myoglobin, 17 kDa. Vo, void volume.

it is rather unlikely that the disruption of the hydrophobic contact made by Trp-120 to biotin alone could cause such a drastic reduction in biotin-binding affinity. Thus, the mutation of Trp-120 to Phe may have generated additional structural changes in or around the biotin-binding site which considerably lower the biotin-binding affinity.

Biotin-Binding Stability of Stv-38. To further understand the biotin-binding characteristics of Stv-38, the biotin-binding stability of Stv-38 was investigated under various conditions. Stv-38 stably retained bound ^{14}C-labeled biotin over a pH range of 1.3 to 11.3 or in the presence of urea up to 6 M at neutral pH, as did natural core streptavidin. By contrast, the addition of free, unlabeled biotin at neutral pH released bound ^{14}C-labeled biotin from Stv-38 (Fig. 5). The amount of released

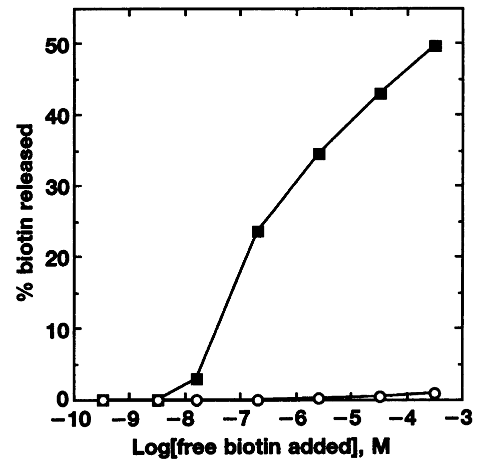

FIG. 5. Release of biotin bound by Stv-38 by the addition of free biotin. Stv-38 was mixed with *d*-[*carbonyl*-^{14}C]biotin at a molar ratio of biotin to biotin-binding site of 1. This Stv-38 solution (1.71 μg; 136 nmol of subunits in 133 μl of TBS) was then mixed with an equal volume (133 μl) of TBS containing various concentrations of free biotin. The mixture was incubated at 21°C for 20 min, transferred to an Ultrafree MC filter (molecular mass cutoff, 10 kDa), and centrifuged at 1600 × *g* for 10 min. The amounts of released radioactive biotin in the filtrate were quantitated by liquid scintillation, and are plotted as a function of the final concentration of free biotin added (■). Natural core streptavidin was also analyzed in the same manner as the control (○).

^{14}C-labeled biotin increased as the concentration of free biotin was raised above 33 nM. Addition of 330 μM free biotin released approximately 50% of the bound ^{14}C-labeled biotin from Stv-38. By contrast, almost no release of bound biotin by natural core streptavidin was observed with the addition of free biotin up to 330 μM. These results demonstrate that Stv-38 retains bound biotin stably, even under relatively harsh conditions, but the addition of free biotin results in the dissociation of previously bound biotin from Stv-38 due, presumably, to exchange of bound biotin with free biotin. In the known three-dimensional structure of streptavidin (14, 15, 33, 35), Trp-120 spatially covers the pentanoyl group of bound biotin. This apparently contributes to the very low dissociation rate constant for streptavidin–biotin complexes (2.8×10^{-6} s^{-1} at pH 7 and 25°C) (3). It is likely, therefore, that the mutation of Trp-120 to Phe would lead to a greater rate constant for the dissociation of bound biotin with minimal effects on the association rate constant, thereby enhancing exchange reactions with free biotin.

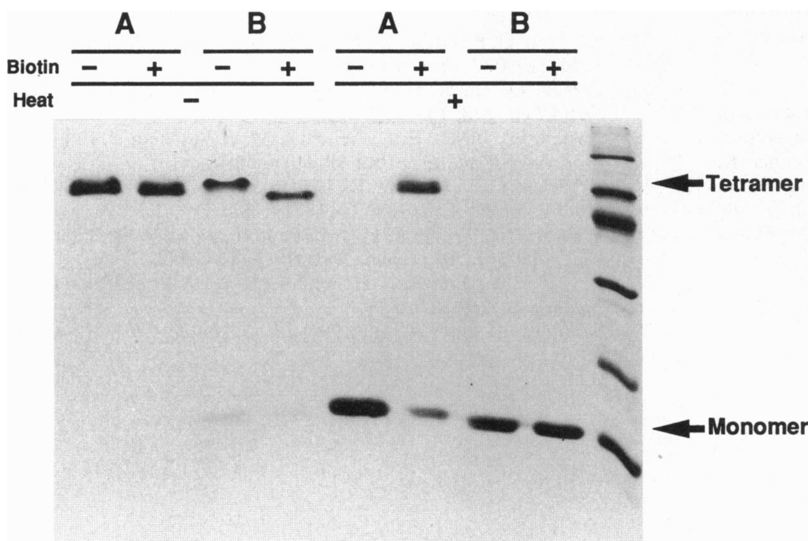

FIG. 6. Effect of biotin binding on subunit association of Stv-38. Natural core streptavidin (5.0 μg; 0.37 nmol of subunits) (*A*) or Stv-38 (4.0 μg; 0.32 nmol of subunits) (*B*), in 4 μl of TBS, was mixed with an equal volume of TBS with (+) or without (−) 1.7 nmol of biotin (molar ratio of biotin to streptavidin subunit, 4.6 for natural core streptavidin and 5.3 for Stv-38), and the mixtures were incubated at 21°C for 5 min. To each of these mixtures (8 μl), 2 μl of 3.0% (wt/vol) SDS/100 mM Tris·HCl, pH 6.8/40% (vol/vol) glycerol were added to a final SDS concentration of 0.6%. The resulting samples were either incubated at 21°C for 5 min (−) or heated in boiling water for 3 min (+) and then subjected to SDS/PAGE.

Effect of Biotin Binding on Subunit Association. Because the hydrophobic contact made by Trp-120 with biotin occurs through the dimer–dimer interface, this intersubunit communication is likely to contribute to the biotin-induced, tighter subunit association of streptavidin, which is observed in natural streptavidin (17). To test this possibility, the subunit association of Stv-38 with and without biotin was investigated by SDS/PAGE (17) (Fig. 6).

Upon heat treatment in the presence of SDS, natural core streptavidin and Stv-38 dissociate completely into monomers in the absence of biotin. However, the dissociation of natural core streptavidin was partly repressed by bound biotin, and two distinct protein bands corresponding to the tetramer and monomer were observed. This result is consistent with our previous observations (17). By contrast, no tetramer band was observed with Stv-38, even in the presence of biotin under the same conditions, indicating that its subunit association was not tightened significantly upon biotin binding. This demonstrated that the intersubunit contact made by Trp-120 to biotin is the primary force which induces the tighter subunit association of natural streptavidin upon biotin binding.

Even without heat treatment, a part of Stv-38 dissociated into monomers in the presence of SDS, while no dissociation was observed with natural core streptavidin. Biotin binding had only a slight effect on the dissociation of Stv-38 without heat treatment. This reveals that Trp-120 also contributes to the subunit association of tetramers in the absence of biotin. Because hydrophobic interactions around the dimer–dimer interface are the major force for stable association of two symmetric dimers, the reduction in hydrophobicity around the dimer–dimer interface, caused by the mutation of Trp-120 to Phe, would also reduce the overall stability of the dimer–dimer association.

Stv-38 as a Biotechnology Tool. The unique properties of Stv-38, with tight and specific, yet reversible, biotin binding and the same tetrameric structure as natural streptavidin, should expand applications of the streptavidin-biotin system. For example, specific purification of biotinylated macromolecules is possible by using immobilized Stv-38, which allows release of captured biotinylated targets simply by the addition of free biotin at neutral pH. Regeneratable macromolecular arrays on solid surfaces by using the streptavidin–biotin interaction could be designed in which surface biotinylated probes could be regenerated or changed, as desired, without the use of harsh conditions. Stv-38 could also be fused to partner proteins to produce chimeras in which the streptavidin moiety provides tight, yet reversible, binding of the partners to biotin, biotin derivatives, and biotinylated macromolecules. Thus, Stv-38 should be able to serve as a biotechnology tool in applications in which irreversible biotin binding by natural streptavidin under the conditions compatible with biological materials is undesirable.

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