The strong biotin-streptavidin interaction limits the application of streptavidin as a reversible affinity matrix for purification of biotinylated biomolecules. To address this concern, a series of single, double, and triple streptavidin muteins with different affinities to biotin were designed. The strategy involves mutating one to three strategically positioned residues (Ser-45, Thr-90, and Asp-128) that interact with biotin and other framework structure-maintaining residues of streptavidin. The muteins were produced in soluble forms via secretion from Bacillus subtilis. The impact of individual residues on the overall structure of streptavidin is reflected by the formation of monomeric streptavidin to different extents. Of the three targeted residues, Asp-128 has the most dramatic effect (Asp-128 > Thr-90 > Ser-45). Conversion of all three targeted residues to alanine results in a soluble biotin binding mutein that exists 100\% in the monomeric state. Both wild-type and mutated (monomeric and tetrameric) streptavidin proteins were purified, and their kinetic parameters (on- and off-rates) were determined using a BIAcore biosensor with biotin-conjugated bovine serum albumin immobilized to the sensor chip. This series of muteins shows a wide spectrum of affinity toward biotin ($K_d$ from $10^{-6}$ to $10^{-11}$ M). Some of them have the potential to serve as reversible biotin binding agents.

Streptavidin is a homotetrameric protein with a biotin binding site in each subunit. The dissociation constant of the streptavidin-biotin complex is estimated to be $4 \times 10^{-14}$ M (1). Because of this tight binding, streptavidin has been widely applied as a capturing molecule for in vitro detection, localization, and immobilization of biotinylated biomolecules (2, 3) and for in vivo imaging, targeting, and drug delivery (4–10). To extend the applicability of streptavidin, reversible interactions between streptavidin and biotinylated molecules may be required under certain conditions. Therefore, it would be useful to develop a series of mutated streptavidin molecules with a wide spectrum of affinity toward biotin. X-ray crystallographic studies (11–14), direct force measurements (15, 16), site-directed mutagenesis (17–21), and construction of circular permuted streptavidin (22, 23) indicate that three major mechanisms contribute to the tight binding between streptavidin and biotin. These include the hydrophobic interactions between biotin and the four tryptophan residues (Trp-79, Trp-92, Trp-108, and Trp-120) in streptavidin, the interaction with a flexible loop (residues 45–52) in streptavidin, and a series of hydrogen-bonding interactions involving eight residues (Asn-23, Ser-27, Tyr-43, Ser-45, Asn-49, Ser-88, Thr-90, and Asp-128) of streptavidin (Fig. 1). Since individual interactions via hydrogen bonding in general are relatively weak (24), replacement of streptavidin residues critical to hydrogen-bonding interactions with biotin by site-directed mutagenesis would allow one to fine tune the binding affinity of streptavidin to biotin. In this study, the roles of key residues involved in hydrogen bonding with the two ureido-nitrogens (Ser-45 and Asp-128) and a sulfur (Thr-90) in biotin were examined. Three single, two double, and one triple streptavidin mutants were constructed, and these mutant proteins (muteins) were produced as soluble proteins via secretion using Bacillus subtilis. Interestingly two (Thr-90 and Asp-128) of three target residues characterized affect not only the interaction between streptavidin and biotin but also the interactions between streptavidin subunits. This leads to the generation of a mixture of tetrameric and monomeric muteins. Combination of all three mutations together results in a triple mutant that produces streptavidin solely in the monomeric form independent of the presence or absence of biotin. Kinetic parameters including on-rate, off-rate, and dissociation constant of each purified mutein and the wild-type control were determined in real time to examine the streptavidin-biotin interaction by using the surface plasmon resonance-based BIAcore biosensor. The results showed that the streptavidin muteins have a wide range of dissociation constants ($10^{-6}$–$10^{-11}$ M). The effects of these mutations on biotin binding and subunit interactions in streptavidin are discussed.

**Experimental Procedures**

**Construction of Streptavidin Mutants—**Construction of streptavidin mutants was based on the principle of cassette mutagenesis using a synthetic streptavidin gene ($ssav$) in the B. subtilis expression vector (pSSAV-Tcry) as the template. $ssav$ encodes the full-length intact form of streptavidin. Different point mutations were introduced to the coding sequence of $ssav$ by using the PCR-based oligonucleotide-directed mutagenesis procedure.

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Production and Purification of Streptavidin—Mutagenic primers for the construction of streptavidin mutants

| Ser45Ala (SM1) | Sacc | 
|---|---|
| Original sequence | 5′...GAT GGA GCT CTT ACA GGA ACA TAT GAA TCT GCT GTT GGA...3′ |
| SAVHNF1 | 5′...GAT GGA GCT CTT ACA GGA ACA TAT GAA CTT GCT GTT GGA...3′ |
| Asp128Ala (SM2) | SacI |
| Original sequence | 5′...G AAAGT ACT CTT GGT GGA CAT GAT ACA TTT AC...3′ |
| SAVDI128AF | 5′...G AAAGT ACT CTT GGT GGA CAT CTT ACA TTT AC...3′ |
| Thr90Ala (SM3) | MscI |
| Original sequence | 5′...GA AAC GCA CAT AGG GCT ACA ACA TGG TCT GGC CAA TAT G...3′ |
| SAVHN1 | 3′...CT TTT CGT GTA TGG CGA CGT GTG ACC AGA CCG GTT ATC...5′ |

Nucleotides that are different from the sequence in the template are indicated in bold and underlined. SAVHNB1 is a backward mutagenic primer, and its sequence is complementary to the original sequence. Translation is based on the original sequence.

Nucleotides that are different from the sequence in the template are indicated in bold and underlined. SAVHNB1 is a backward mutagenic primer, and its sequence is complementary to the original sequence. Translation is based on the original sequence.

Production and Purification of Streptavidin—Expression vectors carrying mutated streptavidin genes were transformed to B. subtilis WB800, an eight protease-deficient strain (26), for streptavidin production via secretion as described previously4 with some modifications. Cells were grown in a semidefined medium, modified casamino acid medium, for 24 h at 32°C and separated from the medium by centrifugation at 10,000 × g for 15 min. The pH of the culture supernatant was corrected to 4.4 by the addition of a 0.5 M succinic acid solution containing 10 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The supernatant was then applied to a cation-exchange column (2.8 × 8 cm) packed with Macro-Prep High S (Bio-Rad) equilibrated with equilibration buffer (100 mM sodium succinate, pH 4.4, 10 mM EDTA, and 1 mM PMSF). The column was washed with 10 bed volumes of equilibration buffer. Bound proteins were eluted with 100 ml of equilibration buffer containing 0.5 M NaCl and dialyzed overnight against 100 mM Na2CO3, pH 9.8, containing 0.5 M NaCl. The dialyzed sample was then applied to a 2-iminobiotin-agarose affinity column (Sigma) equilibrated with 100 mM Na2CO3, pH 9.8, containing 0.5 M NaCl. After washing, streptavidin was eluted with 0.1 M glycine buffer, pH 2.8. The buffer of the pooled fractions was changed to 50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA and 1 mM PMSF by using centrifugal filtering devices (Millipore Corp., Bedford, MA). The purified proteins were used for further analysis.

Separation of Tetrameric and Monomeric Forms of Streptavidin Mutants—For streptavidin mutants that existed in both tetrameric and monomeric forms, fractions containing streptavidin eluted from the 2-iminobiotin-agarose column were applied to a Sephacryl S-100 (2×30 cm) column at 4°C equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl, 10 mM EDTA, and 1 mM PMSF. The tetrameric form was separated from the monomeric form. Fractions for each form were desalted and concentrated by ultrafiltration.

Effect of Biotin on Subunit Association—Purified streptavidin mutants were incubated with 500 μM biotin at room temperature for 30 min and then analyzed by SDS-PAGE and Western blotting.

BIAcoreX Biosensor Study of Streptavidin-Biotin Interaction—Evaluation of kinetic parameters (kₐ, kₐ) was performed by using a surface plasmon resonance-based BIAcoreX biosensor (BIAcore Inc.). In these analyses, BSA was conjugated with biotin and coupled to a CM5 sensor chip. Biotin-conjugated BSA was prepared by incubating 2 mg of BSA with 1 mg of N-hydroxysuccinimide ester of biotin (NHS-biotin, Pierce) in 50 mM sodium carbonate buffer, pH 8.0, at 4°C for up to 2 h. The molar ratio of biotin to BSA under the reaction condition was 60:1. Unreacted NHS and NHS byproducts were removed by ultrafiltration involving three washes with 50 mM sodium carbonate buffer, pH 8.0, and one wash with 50 mM sodium carbonate, pH 6.5. The conjugate was immobilized on the dextran matrix of the sensor chip using the amine coupling kit (BIAcore Inc.) with the procedures specified by the manufacturer. There are two flow cells per sensor chip for the BIAcoreX system. The conjugate was immobilized in the first flow cell (FC-1) to 700 resonance units. BSA was immobilized in the second flow cell (FC-2) to 950 resonance units as a negative control for background correction. Solutions containing streptavidin at different concentrations (20–200 nM) were passed through the flow cells at a flow rate of 10
μl/min. After completion of each run, the sensor chip was regenerated with a 2.5-min pulse of 100 mM NaOH containing 4 M NaCl and 2 μM biotin at a flow rate of 5 μl/min.

**Kinetic Analysis of Streptavidin Muteins**—A three-step scheme was applied to determine the on-rate (association rate constant, $k_a$) for the streptavidin-biotin interaction. Binding of streptavidin to biotin-conjugated BSA immobilized to the sensor chip is reflected by the increase in resonance units with time. In the first step, the change in resonance units with time was plotted at different concentrations of streptavidin injected. This allows the determination of the binding rate (dR/dt) with $R$ representing the resonance unit at each concentration of streptavidin used in the study. In the second step, the binding rates at different concentrations of streptavidin injected were plotted against the residence time. In the third step, the binding rate at each concentration of streptavidin injected was determined based on the equation $\ln(R/R_0) = k_a t - t_0$ (27). This yielded a series of straight lines (one line per concentration of streptavidin injected). The slope of each line (designated $k_a$) represents the value of $k_a$ at each concentration of streptavidin injected. A straight line should be generated, and the slope of this line represents $k_a$ (Fig. 6A).

At the end of injection, the streptavidin sample was displaced by the injection of binding buffer, and the dissociation of streptavidin was monitored for 60 min. The off-rate (dissociation rate constant, $k_d$) was determined based on the equation $\ln(R/R_0) = k_d (t - t_0)$ (27). By plotting $\ln(R/R_0)$ versus $(t - t_0)$, the off-rate ($k_d$) for streptavidin to dissociate from biotin-conjugated BSA was determined (Fig. 6B). All these measurements were performed at 25°C.

**Other Methods**—All PCRs were carried out using Vent polymerase (New England Biolabs). The PCR conditions, plasmid isolation, DNA transformation, Western blotting, and nucleotide sequencing were performed as described previously (28, 29). The concentration of purified streptavidin was determined spectrophotometrically using a molar extinction coefficient of 41,820 M$^{-1}$ cm$^{-1}$ at 280 nm (30) for streptavidin.

The molecular weight of tetrameric and monomeric forms of streptavidin was determined by gel filtration using a Bio-Rad biogel Bio-Gel P-200 column calibrated with protein standards. For viewing the three-dimensional structure of the streptavidin-biotin complex (PDB code 1SWE), the Swiss-Pdb viewer program (31) was used. To analyze the intersubunit contacts and solvent-accessible areas of Ser-45, Thr-90, and Asp-128 in the streptavidin-biotin complex, web-based computer programs (32) including ligand-protein contacts (LPC software) and contacts of structural units (CSU software) were used.

**FIG. 1. Biotin binding pocket of streptavidin.** Residues located within 5 Å from biotin are displayed. The eight residues that form hydrogen bonds (green dotted line) with biotin are labeled with the three target residues examined in this study highlighted in light-blue for Ser-45, red for Thr-90, and yellow-brown for Asp-128. Two tryptophan residues (Trp-92 and Trp-108) that form hydrogen bonds with Asp-128 are also labeled. Biotin is colored in yellow. Trp-79 located at the front is omitted so that the hydrogen bonding network for Asp-128, Asn-23, Trp-92, Trp-108, and biotin can be visualized clearly.

**FIG. 2. Construction of expression vectors for streptavidin muteins.** The restriction sites used in construction (see text) are indicated. P43, a constitutively expressed B. subtilis promoter; SacB SP, B. subtilis levansucrase signal peptide; Tcry, a transcription terminator from the Bacillus thuringiensis crystal toxin gene; SAV, streptavidin; bp, base pair.

**RESULTS**

**Design and Construction of Streptavidin Mutants**—To develop a series of streptavidin muteins with a wide range of affinity toward biotin, the role of three residues located at strategic positions that form hydrogen bonds with biotin was studied (Fig. 1). Asp-128 and Ser-45 interact with two biotin ureido-nitrogen atoms, while Thr-90 interacts with the sulfur atom in biotin. Each of them was changed to alanine to generate three single mutants designated SM1(S45A), SM2(D128A), and SM3(T90A), respectively, via the PCR-based cassette mutagenesis (Fig. 2, A and B) involving a synthetic streptavidin gene. To examine whether the combination of these single mutations together would further reduce the interactions between streptavidin and biotin, two double mutants, DM1(S45A,T90A) and DM2(S45A,D128A), and one triple mutant TM1(S45A,T90A,D128A) were constructed (Fig. 2C).

**Monomeric and Tetrameric Forms of Streptavidin Muteins Produced via Secretion from B. subtilis**—In the expression vector, the B. subtilis levansucrase (SacB) signal sequence was applied to direct the secretion of streptavidin muteins to the culture medium. B. subtilis WB800, an eight extracellular protease-deficient strain, served as the expression host. The use of this host is important since the production yield of the secreted streptavidin muteins is about 2-fold higher in most cases in comparison with the use of WB700, a seven extracellular protease-deficient strain, as the host. Streptavidin and its muteins were secreted as soluble proteins in the culture medium. If the streptavidin-containing culture supernatant was analyzed by
Monomeric Streptavidin Muteins Retained the Iminobiotin Binding Capability—To determine whether different forms of streptavidin muteins still retained the iminobiotin binding ability, a 2-iminobiotin-agarose column (33) was used in the second step of a two-step purification scheme. Without overloading the column, all streptavidin molecules including both the monomeric and tetrameric forms were bound to the column as determined by the SDS-PAGE analysis of the flow-through and the elution fractions. To determine whether the monomeric streptavidin muteins really existed in solution or they were formed because of the presence of SDS under the condition for SDS-PAGE, streptavidin eluted from the 2-iminobiotin column was loaded to a Bio-Prep S.E. 100/17 gel-filtration column. Streptavidin muteins could be resolved into two peaks. The one eluted earlier corresponded to the tetrameric form of the streptavidin muteins from SM2(D128A) and SM3(T90A) were concentrated 23- and 15-fold, respectively, by ultrafiltration. The concentrated samples had a final concentration of 1.86 \( \times 10^{-3} \) M, molecular weight markers; I-SAV, intact (wild-type, full-length) streptavidin; RT, room temperature (unboiled samples were loaded). Tetrameric and monomeric forms are indicated by open and closed arrows, respectively, at the right side.

**TABLE II**

| Designation          | Streptavidin   | %    | \( k_{on} \) \( M^{-1} s^{-1} \) | \( k_{off} \) \( s^{-1} \) | \( K_d \) \( M \) |
|----------------------|----------------|------|----------------------------------|--------------------------|------------------|
| **Wild-type SAV**    |                | 100  | \( 5.13 \times 10^6 \)           | ND                       | ND               |
| **SM1**              | S45A          | 90–100 | \( 2.91 \times 10^6 \)           | \( 9.09 \times 10^{-5} \) | \( 3.12 \times 10^{-11} \) |
| **SM2**              | D128A         | 12–20 | \( 4.12 \times 10^6 \)           | \( 7.88 \times 10^{-5} \) | \( 1.91 \times 10^{-11} \) |
| **SM3**              | T90A          | 80–88 | \( 7.01 \times 10^4 \)           | \( 5.89 \times 10^{-4} \) | \( 8.40 \times 10^{-9} \) |
| **DM1**              | S45A,T90A     | 45–55 | \( 9.93 \times 10^5 \)           | ND                       | ND               |
| **DM2**              | S45A,D128A    | 45–55 | \( 3.38 \times 10^4 \)           | \( 5.75 \times 10^{-5} \) | \( 1.70 \times 10^{-9} \) |
| **TM1**              | S45A,T90A,D128A | 12–20 | \( 1.45 \times 10^5 \)           | \( 2.33 \times 10^{-5} \) | \( 1.60 \times 10^{-11} \) |

*ND, not determined because these off-rates are beyond the measurable range.

*NC, not characterized because of the low abundance of this protein.
tion profiles indicated that the monomeric streptavidin muteins remained in the monomeric state (data not shown). In contrast, wild-type streptavidin remained in the tetrameric state in a concentration-independent manner (0.06–2.3 μg/liter) if these samples were not boiled before loading to SDS-polyacrylamide gels.

**Effect of Biotin on Subunit Association of Streptavidin Muteins**—Biotin binding is known to strengthen the interaction between streptavidin subunits (34, 35). It would be of interest to examine whether biotin can induce monomeric streptavidin muteins to self-assemble into tetrameric form. To some extent, promotion of tetramerization of the monomeric form of streptavidin muteins from SM1(S45A) and SM2(D128A) in the presence of biotin was observed (Fig. 5A). In contrast, the effect of biotin on oligomerization of monomeric SM3(T90A) mutein was insignificant. For double (DM1 and DM2) and triple (TM1) mutants, addition of biotin did not improve tetramerization of these muteins to any significant extent (Fig. 5B).

**Kinetic Parameters of Monomeric and Tetrameric Streptavidin Muteins on Biotin Binding**—To examine the mutagenic effects of S45A, T90A, and D128A in streptavidin in biotin binding, both on-rate and off-rate of the purified forms of wild-type and mutant streptavidin proteins were determined using the surface plasmon resonance-based BIACore biosensor with biotin-conjugated BSA immobilized on the sensor chip (Fig. 6). The data are summarized in Table II. Wild-type streptavidin has an on-rate of 5.13 × 10⁶ M⁻¹ s⁻¹. The off-rate is beyond the measurable limit of the biosensor system since the dissociation reaction is too slow.

For the three single mutants (SM1 to SM3), a general trend concerning the on-rate could be observed depending on whether these muteins existed in the tetrameric or monomeric state. If the muteins existed in the tetrameric state, the on-rates were usually not significantly affected (less than 5.2-fold). In contrast, a more drastic decrease in on-rate could be observed when these muteins existed in the monomeric state. Such a drastic change in on-rate reflected some structural changes in the subunit that led to poor subunit interactions. For the off-rates, monomeric muteins also consistently showed a higher off-rate than their respective tetrameric muteins.

In the case of tetrameric SM1(S45A), the observed increase in the dissociation constant was mainly because of the increase in the off-rate. For both SM2(D128A) and SM3(T90A), these muteins existed in both monomeric and tetrameric forms. The increase in off-rate of the tetrameric muteins reflected more closely the importance of Asp-128 and Thr-90 in hydrogen bonding with biotin in these muteins. Among the three residues examined, the impact of Thr-90 in hydrogen bonding with biotin was less than Ser-45 and Asp-128. In the monomeric mutants, the impact of Thr-90 in hydrogen bonding with biotin is less significant (Fig. 5). Changing one or more of these residues allowed us to examine whether biotin can induce monomeric streptavidin from mutants with double and triple mutations or without (−) 500 μM biotin at room temperature for 30 min and then analyzed by Western blotting. A, streptavidin muteins from mutants with single mutation; B, streptavidin muteins from double and triple mutations. M, molecular weight markers; I-SAV, intact (wild-type, full-length) streptavidin; RT, room temperature. Tetrameric and monomeric forms are indicated by open and closed arrows, respectively, at the right side.

**FIG. 4. Determination of molecular weight of tetrameric and monomeric streptavidin by gel filtration.** The purified and concentrated sample of streptavidin was injected in a Bio-Rad biologic work station with a Bio-Prep S.E. 100/17 gel-filtration column. Vₑ and Vₑ represent the elution volume and the void volume for the column, respectively. The column was calibrated with molecular mass protein standards as shown for equilibration and elution, a 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA and 1 mM PMSF was used. A, sample from SM3(T90A); B, sample from SM2(D128A).

**FIG. 5. Effect of biotin binding on subunit association of streptavidin muteins.** The individual mutant strain was grown in modified casamino acid medium, and secreted streptavidin was purified. Purified proteins were incubated with (+) or without (−) 500 μM biotin at room temperature for 30 min and then analyzed by Western blotting. A, streptavidin from mutants with single mutation; B, streptavidin from mutants with double and triple mutations. M, molecular weight markers; I-SAV, intact (wild-type, full-length) streptavidin; RT, room temperature. Tetrameric and monomeric forms are indicated by open and closed arrows, respectively, at the right side.
develop a series of streptavidin muteins with a wide range of affinity toward biotin.

To examine the functional roles of a particular residue in streptavidin that interacts with biotin, it is a common practice to replace such residue with alanine via site-directed mutagenesis (17–21). In most of the cases, such changes would not affect the gross structure of the protein. In this study, three different residues in streptavidin were individually changed to alanine, and each one showed a different degree of impact (i.e. D128A > T90A > S45A) on the ability of these muteins to tetramerize. Analysis of residue contacts and solvent-accessible area of streptavidin-biotin complex provided insights that correlate with our observations. Among all three residues analyzed, Asp-128 has the largest buried surface area in the complex. It interacts with 10 neighboring residues by hydrogen bonding, electrostatic, and van der Waals interactions with a total of 53 interatomic contacts. Many interacting residues are important either to form hydrogen bonding with biotin (e.g. Asn-23) or to form the hydrophobic lining (e.g. Trp-92 and Trp-108) of the biotin binding pocket (Fig. 1). They also interact with other residues in maintaining the framework structure of streptavidin. Missing these vital contacts and creation of a small cavity in SM2 could alter the structure in such a way that it could affect the subunit interactions. Threonine 90 has the second largest buried area in the complex and has a total of 44 atomic contacts including a few key residues (Trp-79, Trp-92, and Trp-108) that form the hydrophobic pocket for biotin binding (18). Among the three target residues analyzed, serine 45 has the smallest buried area in the complex. Some of the interatomic interactions are little affected by the replacement with an alanine side chain (19). This may explain why SM1(S45A) could maintain the overall tetrameric structure.

To form a biotin binding, monomeric streptavidin in the soluble form has been a great challenge. Hydrophobic surfaces involved in subunit interactions are exposed in the monomeric form, and a decrease in solubility of monomeric streptavidin can pose a problem. Two versions of engineered dimeric forms of streptavidin are indeed reported to have low solubility (36). An extensive replacement of hydrophobic residues in the interfaces with hydrophilic residues may solve this problem. Successful production of monomeric avidin molecules has recently been reported (37). The scheme involves the replacement of three hydrophobic residues at the 1-3 interface to improve the solubility and one polar residue at the 1-4 interface to reduce the hydrogen-bonding interaction. However, in the presence of biotin, these monomeric avidin molecules reassociate into the tetrameric form. To generate a monomeric streptavidin would be even more difficult since the subunit interactions in streptavidin are even stronger than those in avidin (34). In the present study, each mutation of the three targeted residues generated monomeric streptavidin to a different extent; a combination of these mutations would provide a means to develop monomeric streptavidin. This approach was encouraged by the observation that the biotin-mediated tetramerization of monomeric streptavidin was lost (Fig. 5B) for the double muteins (DM1 and DM2). More important, a combination of all three mutations in TM1 allows the development of a monomeric streptavidin (100%) in its soluble form with biotin binding capability. This represents a true monomeric streptavidin since it would not oligomerize back to the tetrameric state even in the presence of biotin. It is important to note that not a single surface hydrophobic residue at the interface was replaced in TM1. This illustrates that changes of the internal structure of streptavidin can lead to structural changes at the surface of the subunit in such a way that it no longer interacts with other subunits but still remains soluble in solution. X-ray crystallographic studies of TM1 would provide insightful information concerning the structural changes of this protein.

Although streptavidin has been well characterized, the on-rate for biotin binding and the dissociation constant of the streptavidin-biotin complex commonly cited in the literature can be used as an approximation only. The dissociation constant was derived based on the on-rate of $7 \times 10^7$ M$^{-1}$ s$^{-1}$ and the off-rate of $2.8 \times 10^{-6}$ s$^{-1}$ (1). This on-rate, in fact, is based on the value for the avidin-biotin interaction rather than the streptavidin-biotin interaction (1). Our study determined and reported for the first time the on-rate for the streptavidin-biotin interaction. In general, the on-rate of a binding reaction depends on several parameters including diffusion coefficients of the two interacting molecules, desolvation of solvent during the binding reaction, and conformational changes needed for a stable interaction. Since a typical diffusion-controlled binding reaction for biomolecules has the on-rate in the range of $10^9$–$10^9$ M$^{-1}$ s$^{-1}$ (38), the on-rate ($5 \times 10^9$ M$^{-1}$ s$^{-1}$) for the streptavidin-biotin interaction indicates that this binding is not a diffusion-controlled reaction even though one of the ligands (biotin) in the binding reaction is relatively small in size. This finding is consistent with the x-ray crystallographic data of the streptavidin-biotin complex from three aspects. First, the exposed surface area of the four biotin binding sites in a tetrameric streptavidin represents $\sim2\%$ of the total exposed sur-
face area of a tetrameric streptavidin. Therefore, not every collision between streptavidin and biotin can result in a productive binding. Second, desolvation of five bound water molecules in each biotin binding site was required during the binding reaction. Third, a flexible loop in streptavidin becomes immobilized after biotin binding and closes the biotin binding pocket (23). All these factors together can reduce the on-rate of a diffusion-controlled reaction to an on-rate in the range of $10^6 \text{M}^{-1}\text{s}^{-1}$. In comparison with avidin, the higher dissociation constant of the streptavidin-biotin complex is contributed by both a higher off-rate (1) and a lower on-rate.

The value of the off-rate of the streptavidin-biotin complex has been reported to be $2.8 \times 10^{-6} \text{ s}^{-1}$ (1) based on the exchange reaction at 25°C. Because of the slow dissociation process, our BIACore measurement could not generate an off-rate for the streptavidin-biotin complex. However, the biosensor allows the measurement of an off-rate as low as $1 \times 10^{-5} \text{ s}^{-1}$. The reported off-rate of $2.8 \times 10^{-6} \text{ s}^{-1}$ indeed falls into a nonmeasurable range for the biosensor system. If the off-rate of $2.8 \times 10^{-6} \text{ s}^{-1}$ is used in our calculation, the dissociation constant for the streptavidin-biotin complex at 25°C is estimated to be $5.5 \times 10^{-13} \text{ M}$.

All monomeric streptavidin muteins showed a significant increase in the dissociation constant. The increase was contributed by both a significant decrease in the on-rate and an increase in the off-rate. A drastic change in both on-rate and off-rate in part reflects a structural change that makes biotin binding not as tight as the wild-type streptavidin. To form a complete biotin binding site in a streptavidin subunit, a tryptophan residue (Trp-120) from a neighboring subunit is required. Mutation of Trp-120 in streptavidin to alanine, lysine, and Asp-128 with their interacting residues. These, if not all, would be the major factors that accounted for the changes in the dissociation constants. For the monomeric TM1 (S45A,T90A,D128A) mutein, the dissociation constant increased further to $1.7 \times 10^{-6} \text{ M}$.

Monomeric muteins (DM1, DM2, and TM1) with dissociation constants in the range of $10^{-5}$ to $10^{-8} \text{ M}$ could potentially be applied to develop affinity matrices to purify biotinylated molecules since the interaction between muteins and biotin would be reversible. Currently the common approach to purifying biotinylated proteins is the use of the monomeric avidin matrix (25, 40). The dissociation constant for this system is in the range of $10^{-7} \text{ M}$. This matrix is generated by a multistep process that requires denaturation and renaturation of the immobilized avidin. Availability of an alternative that requires more simple manipulations is definitely desirable.

While our streptavidin mutein project was in progress, two streptavidin muteins D128A (21) and S45A (19) were independently constructed. These muteins were produced in the form of inclusion bodies in E. coli. Refolding through a denaturation and renaturation cycle was required to generate streptavidin muteins in the soluble form. All the refolded muteins were reported to be tetrameric and could maintain the overall structure as reflected by x-ray crystallographic studies. In the present study, which did not require any refolding of the muteins, we observed the presence of a high percentage of the SM2(D128A) mutein in the monomeric form. Our muteins are also different from those developed by others (19, 21) in the kinetic properties (off-rates and dissociation constants). These differences may be due to the use of different methods in sample preparation (secretion versus refolding) and the use of different approaches (biosensor versus calorimetry) and conditions to obtain the kinetic data.

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