Engineering Soluble Monomeric Streptavidin with Reversible Biotin Binding Capability* S

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Sau-Ching Wu and Sui-Lam Wong‡
From the Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Monomeric streptavidin with reversible biotin binding capability has many potential applications. Because a complete biotin binding site in each streptavidin subunit requires the contribution of tryptophan 120 from a neighboring subunit, monomerization of the natural tetrameric streptavidin can generate streptavidin with reduced biotin binding affinity. Three residues, valine 55, threonine 76, and valine 125, were changed to either arginine or threonine to create electrostatic repulsion and steric hindrance at the interfaces. The double mutation (T76R,V125R) was highly effective to monomerize streptavidin. Because interfacial hydrophobic residues are exposed to solvent once tetrameric streptavidin is converted to the monomeric state, a quadruple mutein (T76R,V125R,V55T,L109T) was developed. The first two mutations are for monomerization, whereas the last two mutations aim to improve hydrophilicity at the interface to minimize aggregation. Monomerization was confirmed by four different approaches including gel filtration, dynamic light scattering, sensitivity to proteinase K, and chemical cross-linking. The quadruple mutein remained in the monomeric state at a concentration greater than 2 mg/ml. Its kinetic parameters for interaction with biotin suggest excellent reversible biotin binding capability, which enables the mutein to be easily purified on the biotin-agarose matrix. Another mutein (D61A,W120K) was developed based on two mutations that have been shown to be effective in monomerizing avidin. This streptavidin mutein was oligomeric in nature. This illustrates the importance in selecting the appropriate residues and approaches for effective monomerization of streptavidin.

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‡ To whom correspondence should be addressed: Dept. of Biological Sciences, Division of Cellular, Molecular and Microbial Biology, University of Calgary, 2500 University Dr., N.W. Calgary, Alberta T2N 1N4, Canada. Tel.: 403-220-5721; Fax: 403-289-9311; E-mail: slwong@ucalgary.ca.

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Experimental Procedures

Construction of Streptavidin Mutants—Different point mutations were introduced to the coding sequence of a synthetic streptavidin gene (sava) in the B. subtilis expression vector pSSAV-Tery (14) by PCR-
Engineering of Monomeric Streptavidin

Mutagenic primers for the construction of streptavidin mutants

| Mutated codons | Forward primer | Backward primer |
|----------------|----------------|----------------|
| V125R,V125T (Scal/SphI) | SAVV125RTP | 5’-GGGAGCTGTTTTTCCATGCATTACTTGTTGTTCCAGATGTTAA |
| V55R,V55T (XbaI/SphI) | SAVV55RTP | 5’-CAAGAGTACTTTTGCACTTGTTACAGGAAGATAT |
| V125R,V125T (Scal/SphI) | SAVV125RTP | 5’-GGGAGCTGTTTTTCCATGCATTACTTGTTGTTCCAGATGTTAA |
| T76R (BamHII/SphI) | SAVT76RF | 5’-GTGGATCCGGAACAGCACTTGGATGG |
| D61A,W120K (XbaI/ScaI) | SAVD61AF | 5’-AGAATCTAGATACGTGCTTACAGGAAGATAT |
| T76R,L109T (BamHII/ScaI) | SAVT76RF | 5’-GGGAGCTGTTTTTCCATGCATTACTTGTTGTTCCAGATGTTAA |

Based oligonucleotide-directed mutagenesis. Five mutants (V125R, V125T, V55R, V55T, and T76R), each bearing a single mutation that results in the change of an amino acid residue as the name suggests, were constructed using pSSAV-Tcry as the template and the primers listed in Table I. The amplified products were digested with the pair of enzymes listed in Table I and cloned into pSSAV-Tcry. Five plasmids (pV125R, pV125T, pV55R, pV55T, and pT76R) resulted.

Two double mutants (M2 and AK) were also constructed. For M2 (T76R,V125R), a Scal/Nhel-digested fragment of pV125R was used to replace the corresponding fragment in pT76R. For AK (D61A,W120K), the fragment bearing the two mutations was amplified by PCR using the primers SAVD61AF and SAVW120KB (Table I) and the template pSSAV-Tcry. The amplified fragment was digested by XbaI/ScaI and used to replace the corresponding fragment in pSSAV-Tcry.

The construction of M4 (T76R,V125R,V55T,L109T) involved two steps (Supplemental Fig. S1). First, a 164-bp fragment bearing two mutations (T76R,L109T) was amplified using SAVT76RF and SAVL109TB (Table I) as primers and pSSAV-Tcry as template. The amplified product was digested by BamHI/ScaI and used to replace the corresponding fragment in pV55T-V76R-L109T. In the second step, a Scal/Nhel-digested fragment of pV125R was used to replace the corresponding fragment in pV55T-V76R-L109T to generate pV55T-V76R-L109T-V125R.

Production and Purification of Streptavidin—Wild-type streptavidin was produced by E. coli (strain Bl21 DE3). E. coli [BL21 (DE3)] harboring plasmids expressing streptavidin (pSSAV-Tcry) were grown in a defined medium (14). The secreted protein was purified to homogeneity using a CM5 sensor chip in the Sting Millennium Suite (22). The plots and align the structures of streptavidin and avidin. Interfacial contact areas were calculated using the protein-protein interaction server (21) and the Formiga module in the Sting Millennium Suite (22). The plots of accessible surface area of individual residues in streptavidin in either the monomeric or tetrameric state were generated using the Protein Dossier module in the Sting Millennium Suite.

RESULTS

Selection of Key Residues in Streptavidin for Site-directed Mutagenesis—Tetrameric streptavidin is arranged as a dimer of dimers (Fig. 1A). The interface between subunits A and B (and between C and D) has the most extensive subunit interactions. The interfacial contact area between A and B is 1,557 Å² with 17 H-bonding interactions, two salt bridges, and numerous van der Waals interactions. The interfacial contact area between A and D is also extensive with a contact area of 525 Å² and two interfacial H-bonding interactions. The weakest interface interaction is between subunits A and C with an interfacial contact area of 171 Å². To engineer monomeric streptavidin with a minimal number of mutated residues, an attractive approach is to introduce both charge repulsion and steric hindrance at these interfaces. As protein has structural plasticity (23–25), it is vital to select interfacial residues located on a rigid surface to maximize the effects of charge repulsion and steric hindrance. Because streptavidin subunit forms an eight-antiparallel stranded β-barrel structure (4, 5), the selected containing 5 mM CaCl₂, pH 8.0. The reaction was stopped by precipitation with trichloroacetic acid (18). Boiled samples of precipitated proteins were resolved by reducing SDS-PAGE. The same analysis was performed with streptavidin samples treated with biotin (1 mM final concentration) prior to proteinase K digestion.

Cross-linking Reactions—Cross-linking of streptavidin and its muteins was carried out using ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS) (Pierce) as the cross-linker. A typical reaction mixture (20 μl) contained the purified mutein (0.25 mg/ml) and sulfo-EGS (10-fold molar excess over the protein) in PBS. After 30 min at room temperature, the reaction was quenched with Tris-HCl (30 mM, pH 7.5). Aliquots of the cross-linking reaction samples were boiled and examined by SDS-PAGE. Lysozyme (Sigma, 0.25 mg/ml) was included in the study to help establish the optimal reaction conditions.

Kinetic Analysis of Streptavidin Mutants—The kinetic parameters (both on and off rates for interaction with biotin) of streptavidin muteins were determined in real time using the surface plasmon resonance-based BIAcoreX biosensor. Biotin-conjugated bovine serum albumin immobilized on a CM5 sensor chip was used to study the reversibility of biotin binding (12).

Computer Programs for Streptavidin Analyses—Swiss-pdb Viewer (19) was used to display streptavidin (Protein Data Bank code 1SWE (20)), analyze interfacial residues, measure distance between residues, and align the structures of streptavidin and avidin. Interfacial contact areas were calculated using the protein-protein interaction server (21) and the Formiga module in the Sting Millennium Suite (22). The plots of accessible surface area of individual residues in streptavidin in either the monomeric or tetrameric state were generated using the Protein Dossier module in the Sting Millennium Suite.

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residues should be located on the β-strands rather than in the loop regions. Furthermore, the selected residue in one subunit should be located very close to the equivalent residue or a charged residue in another subunit at the interface. Examination of interfacial residues (Fig. 1, B–D) shows that Thr-76, Val-125, and Val-55 meet the criteria. Hence, they were selected for mutagenesis.

Effects of Single Mutations on Monomerization of Streptavidin—Streptavidin muteins carrying a single amino acid change at the selected site were produced in their soluble form by B. subtilis via secretion. Analysis of non-boiled culture supernatants by SDS-PAGE offers a quick screen for the mutation effect (12). Weaker subunit interaction would result in a higher percentage of the sample in the monomeric state on the SDS-polyacrylamide gel. Because biotin can strengthen subunit interaction, samples were analyzed in the presence or absence of biotin (9, 26). The impact of the mutation on weakening of the subunit interaction followed the order: T76R > V125R > V125T ∼ V55R > V55T (Fig. 2 and Table II). The T76R mutein (designated M1) existed 100% in the monomeric state on the SDS-polyacrylamide gel even in the presence of biotin. In contrast, V55T mutation had the lowest impact with the majority of molecules in the tetrameric state even in the absence of added biotin. Presence of biotin shifts the majority of the three remaining muteins (V125R, V125T, and V55R) to the tetrameric state. As expected, changing valine to arginine exerted greater impact than changing it to threonine. This is true for both Val-125 and Val-55.

Effects of Multiple Mutations on Monomerization of Streptavidin—To develop idealized monomeric streptavidin muteins that are more likely to remain in the monomeric state at high streptavidin concentrations and have excellent reversible biotin binding capability, two more muteins were created. M2 is the double mutant carrying both the T76R and V125R mutations. M4 is a quadruple mutant carrying T76R, V125R, V55T, and L109T mutations. In this combination, the three interfacial hydrophobic residues Val-125, Val-55, and Leu-109 were changed to hydrophilic ones. The last construct is AK, a double mutant (D61A, W120K) carrying two mutations (equivalent to those performed in avidin) that have been shown to convert...
tetrameric avidin to the monomeric state (10). As shown in Fig. 3 and Table II, just like M1, all these muteins existed in monomeric state on the SDS-polyacrylamide gel even in the presence of biotin.

Purification of Streptavidin Muteins—Purification of M4 was used as an example to illustrate the process (Fig. 4). Proteins partially purified by ion exchange chromatography (lane 2) were applied to a biotin-agarose column. M4 could be readily eluted off from the column using biotin-containing buffer as the eluant (lanes 5–7). Pure streptavidin mutein obtained by this simple procedure, after removal of biotin by dialysis, could be used for biochemical characterizations. To demonstrate that dialysis could effectively remove any bound biotin from the M4 mutein, the dialyzed sample was reloaded to the biotin-agarose matrix. Over 95% of the sample could be retained on the column and eluted off from the column using biotin (data not shown). Of all the muteins, M1 tended to have a long trailing arm during elution. This indicates that M1 may not have the desirable reversible biotin binding property. Therefore, it was not characterized further.

Determination of Apparent Molecular Mass of Streptavidin Muteins by Gel Filtration—Observation of 100% monomerization of the streptavidin mutein using a non-boiled sample for SDS-PAGE does not always truly reflect its existence in the monomeric state in solution because SDS can promote subunit dissociation (27, 28). The apparent molecular masses of the purified wild-type streptavidin and the three muteins (M2, M4, and AK) were estimated by gel filtration (Supplemental Fig. 2A and Table III). The expected molecular mass of monomeric streptavidin is 16.5 kDa. M2 and M4 in the absence of biotin showed the apparent molecular masses of 19.95 and 21.87 kDa, respectively. These masses increased slightly in the presence of biotin. These data suggest that the muteins are monomeric in nature because their masses are less than that for the streptavidin dimer (33 kDa). In contrast, the AK mutein showed an apparent molecular mass of 45.66 kDa even in the absence of biotin. This indicates the oligomeric nature of this mutein. Supplemental Fig. 2B shows the elution profile of purified M4 (in the absence of biotin) from the gel filtration column. The sample (loaded at 2 mg/ml) was eluted as a single peak. There is no evidence for the presence of tetrameric streptavidin, which would be eluted at 30.5 min.

**Determination of Apparent Molecular Mass of Streptavidin Muteins by Dynamic Light Scattering**—Because the apparent molecular mass of wild-type streptavidin in the absence of biotin is 10 kDa less than expected (56 instead of 66 kDa) as determined by gel filtration, dynamic light scattering (29) was used as a second method to estimate the apparent molecular masses. The apparent molecular mass of wild-type streptavidin obtained in this way (69 kDa) was closer to that expected (66 kDa) (Table III). The apparent molecular masses for both M2 and M4 in the absence of biotin indicated that they were in the monomeric state. Addition of biotin caused only a slight increase in their apparent molecular masses. The AK mutein again was found to be oligomeric independent of the presence or absence of biotin.

**Proteinase K Sensitivity of Streptavidin Muteins**—Monomeric streptavidin is expected to be more susceptible to proteinase K digestion (10). Therefore, wild-type streptavidin and its muteins were treated with proteinase K (Fig. 5A). Wild-type streptavidin was converted to the core form independent of the presence or absence of biotin. Under the condition used, the core streptavidin was resistant to further degradation by proteinase K. In contrast, all three muteins including AK, M2, and M4 were much more susceptible to proteinase K digestion. Sensitivity to proteinase K is more apparent for M2 and M4, which were completely digested independent of the presence or absence of biotin. This property is consistent with the monomeric nature of these muteins. The AK mutein behaved differently. Although most of it was digested by proteinase K in the absence of biotin, it became much more resistant to proteinase K when biotin was present.

**Cross-linking of Streptavidin and Its Muteins**—To strengthen the idea that both M2 and M4 are monomeric whereas AK is oligomeric in nature, protein cross-linking was carried out using sulfo-EGS as the cross-linking agent. Sulfo-EGS reacts with both the accessible ε-amino groups at the N termini and the surface-exposed ε-amino groups of the lysine side chains in proteins. Secreted wild-type streptavidin has eight lysine residues in each subunit. The three-dimensional structural model of streptavidin suggests that lysine 121 in subunit A is 14.1 Å from lysine 121 in subunit D. As the spacer arm in sulfo-EGS is 16.1 Å, subunits A and D (same for subunits B and C) should be easily cross-linked by sulfo-EGS. Also it is possible to have cross-linking between subunits A and B as the N-terminal region from subunit A, which contains two lysine residues, is likely to be positioned close to lysine 80 in subunit B. The same is true for subunits C and D. Therefore, one should be able to differentiate tetrameric streptavidin from the monomeric form with the observation of cross-linked tetrameric streptavidin using sulfo-EGS. Lysozyme, well known to be monomeric in solution (30, 31), served as the negative control. Fig. 5B shows that the amount of dimeric lysozyme increased slightly in the presence of the cross-linking agent. This helped set the upper limit of the concentration of sulfo-EGS to be used under the experimental condition. The wild-type streptavidin subunit had an apparent molecular mass of 19 kDa on the SDS gel. After treatment with sulfo-EGS, most of these subunits were cross-linked to dimers and higher oligomers with small amounts remaining in the monomeric state. M2 and M4 muteins behaved very similarly (data for M2 are not shown). The majority of the M2 and M4 muteins after the cross-linking treatment migrated as monomers with small amounts in the dimeric form. These dimers may represent cross-linked monomeric subunits that were artificially generated in the same manner as with lysozyme. AK showed a cross-linking profile very similar to that of the wild-type streptavidin. These data strongly support the idea that M2 and
M4 muteins are monomeric, whereas the AK mutein is oligomeric in solution.

Reversible Interaction between Streptavidin Muteins and Biotin—The on rate and off rate of the interactions between streptavidin muteins and biotin were determined by surface plasmon resonance-based BLAcore biosensor (12). As shown in Table IV (graphical plots for M4 are shown in Supplemental Fig. S3), M2, M4, and AK had their dissociation constant ($K_d$) in the range of $10^{-7}$ M. The off rates ($k_o$) for these muteins were almost the same, whereas the on rate ($k_o$) for the AK mutein was slightly lower than the rest. One of the factors affecting the on rate is the diffusion coefficient (or molecular mass) of the streptavidin molecule. Because AK is oligomeric in nature, this may account for the lower on rate for this mutein-biotin interaction.

**DISCUSSION**

Although streptavidin and avidin have similar three-dimensional structures and biotin binding properties, development of monomeric streptavidin is much more challenging for two reasons. First, streptavidin has stronger subunit interface interactions than avidin (27, 28). More potent mutations are required to weaken this strong interface interaction. Second, monomerization of streptavidin may result in the surface exposure of hydrophobic residues that normally would be buried at the interface in tetrameric streptavidin. This can potentially affect the solubility of the monomeric streptavidin and lead to reassociation of the monomers. The problem can be less dramatic for avidin, which is a glycosylated protein with a carbohydrate chain in each of the avidin subunits.

Despite the challenge, our study illustrates that, by selecting a critical residue located on a rigid surface for mutagenic study at the interface, a single mutation (T76R) can be greatly effective in developing monomeric streptavidin. Besides the suggestion from SDS-PAGE analysis, gel filtration study of the M1 mutein also indicated that the majority of M1 was eluted at a position corresponding to the monomeric form (data not shown). The main drawback for this mutein is its elution behavior on the biotin-agarose column. The elution profile had a typical long trailing tail. Furthermore, more M1 could be recovered by soaking the column overnight with buffer containing biotin. This suggests that some of the M1 population have higher affinity to the matrix.

To increase the efficiency of streptavidin monomerization, M2 mutein was developed by combining two potent mutations (T76R,V125R). Data from gel filtration study, dynamic light scattering, sensitivity to proteinase K, and cross-linking reac-
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FIG. 5. Determination of the monomeric or oligomeric states of wild-type streptavidin and its muteins. Pictures show the Coomassie Blue-stained SDS-polyacrylamide gel. A, proteinase K digestion. B, cross-linking study using sulfo-EGS as the cross-linker. All samples were boiled prior to loading. M, molecular weight markers; wt, wild-type streptavidin; L, lysozyme. Numbering represents streptavidin molecules in monomeric (1), dimeric (2), and oligomeric (3-5) states, respectively.

| Protein | Proteinase K digestion | Crosslinking agent |
|---------|------------------------|-------------------|
|         | No biotin | + biotin | No biotin | + biotin |
|         | Sample | M wt | + AK M2 M4 | M wt | + AK M2 M4 | M L wt | AK M4 |
| M2      | 97        | 65    | 21        | 31    | 26        | 96     | 69     |
| M4      | 87        | 45    | 14        | 31    | 26        | 95     | 67     |
| AK      | 74        | 45    | 14        | 31    | 26        | 95     | 67     |

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