Homotetrameric chicken avidin that binds four molecules of biotin was converted to a monomeric form (monoavidin) by mutations of two interface residues: tryptophan 110 in the $1 \rightarrow 2$ interface was mutated to lysine and asparagine 54 in the $1 \rightarrow 4$ interface was converted to alanine. The affinity for biotin binding of the mutant decreased from $K_d \sim 10^{-15}$ M of the wild-type tetramer to $K_d \sim 10^{-7}$ M, which was studied by an optical biosensor IAsys and by a fluorescence spectroscopical method in solution. The binding was completely reversible. Conversion of the tetramer to a monomer results in increased sensitivity to proteinase K digestion. The antigenic properties of the mutated protein were changed, such that monoavidin was only partially recognized by a polyclonal antibody whereas two different monoclonal antibodies entirely failed to recognize the avidin monomer. This new monomeric avidin, which binds biotin reversibly, may be useful for applications both in vitro and in vivo. It may also shed light on the effect of inter-subunit interactions on the binding of ligands.

Chicken avidin and bacterial streptavidin are structurally analogous proteins, which are well known for the uniquely tenacious affinity they exhibit toward their ligand biotin. This affinity is the firmest protein-ligand interaction known in nature ($K_d \sim 10^{-15}$ M), and, under physiological conditions, the binding can essentially be considered irreversible (1). This tight interaction has been utilized during the past three decades for various applications in the life sciences to purify, probe, and target various materials both in vitro and in vivo (2).

The scientific merit of mutation studies of avidin and streptavidin has been 2-fold. On the one hand, mutations of designated residues have provided important structural and functional information regarding the native protein. On the other, the mutant proteins themselves can be of applicative value for use in avidin-biotin technology, since they frequently exhibit special and sometimes surprising binding properties or physicochemical characteristics. In this context, the affinity of the native biotin-binding protein may be too high for some applications. In some cases, for example, a reversible binding might be desired, e.g. for affinity chromatography where the facile release of biotinylated material from an avidin column is required.

Several reports have demonstrated clearly that in most cases the strong affinity of avidin or streptavidin for biotin is dependent upon the tetrameric architecture of the protein. Indeed, long before the structures of avidin (3, 4) and streptavidin (5, 6) were known, it was recognized that the avidin monomer shows a highly reduced affinity constant, which can be recovered upon conversion of the monomer to the tetrameric state (7). The crystal structures of avidin and streptavidin have revealed that the major reason for this common property of the two biotin-binding proteins is the cross-subunit insertion of a conserved tryptophan residue (Trp-110 in avidin and Trp-120 in streptavidin) from one monomer into the binding site of its neighbor. Mutation of this tryptophan in either protein has resulted in both a reduction of binding affinity toward biotin and a reduction in the stability of the tetramer (8–10).

The quaternary structure of avidin and streptavidin has long been viewed as a dimer of dimers (11) and the transition from tetramer to dimer to monomer has always been intriguing. To study the tetrameric architecture further, we have previously probed the interface interactions in avidin by site-directed mutagenesis. In one study, we mutated the critical 1 $\rightarrow 2$ interface residue, Trp-110 of avidin and the conserved Trp-120 of streptavidin, to lysine, which led to the production of dimeric avidin and streptavidin mutants that bound biotin reversibly (8). In another study, we mutated the critical 1 $\rightarrow 4$ interface residue, asparagine 54, to alanine, which also led to destabilization of tetramer (12). One of the surprising outcomes of that study was the production of avidin mutants that, in the absence of biotin, appeared as monomers in solution, whereas upon binding the vitamin the monomers formed stable tetramers. It was thus of interest to investigate whether the combination of these two types of mutation might lead to a stable avidin monomer.

In the present communication, we describe a monomeric avidin mutant (monoavidin), in which Trp-110 was converted to lysine and Asn-54 was mutated to alanine. The resultant mutant binds biotin specifically in a reversible manner and remains in the monomeric state even upon binding to biotin. The resultant monomeric avidin may have applicative advantages over previously described native and mutated forms of the tetrameric and dimeric avidins and streptavidins for affinity-based separations of biotinylated biomolecules.
Monomeric Avidin

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses—A modified (Invitrogen) donor BacToBac™ Baculovirus Expression System plasmid containing the W110K mutation (8) of avidin cDNA was further mutagenized by adding an N54A substitution by the mega-primer method (13). Subcloning of the PCR-amplified insert was performed as earlier reported by Laitinen et al. (12). A recombinant baculovirus capable of producing the N54A+W110K double mutant in avidin was constructed according to the instructions of the BacToBac™ Baculovirus expression system kit.

Production and Purification of Mutant Avidin—Mutant avidin was produced in Baculovirus-infected insect cells as described previously (14). Purification on a biotin-agarose column was performed from cell extracts grown in biotin-free medium according to Laitinen et al. (8).

Biotin-binding Assays—Reversibility of biotin binding was determined by competitive binding to biotinylated biosensor surfaces, and the determination of binding constants for the mutant avidin to biotin were performed using an IAsys optical biosensor as reported earlier (8). Fluorescence spectroscopy measurements were performed using a PerkinElmer LS55 luminescence spectrometer. The excitation wavelength of 280 nm was used, and the emission spectra was obtained from 320 to 420 nm using 2.5 nm excitation and 17 nm emission slitswidths. An average of four scans with scan at speed 240 nm/min was used to calculate the final result. The protein concentration in this analysis was 20 μg/ml, and the biotin was added in small volumes to final concentrations from 0.02 μM to 15 μM. The protein sample was thoroughly mixed in a cuvette with a micropipette and incubated for 5 min covered from UV light before scanning. The measurements were performed at 23 °C in phosphate-buffered saline containing 1 μM NaCl. The measured spectra were integrated, and the intensities were plotted against biotin concentration. Binding curve (quenching (%)) = a[Btn]/(K_d + [Btm]) + c), where a and c are scaling factors, was fitted to this data using the least square method.

Structure Analyses—The molecular mass of the mutant was calculated from the known amino acid composition using the GCG package program Peptidesort (Genetic Computer Group, Madison, WI). SDS-PAGE, immunoblot analyses, and assays for protease sensitivity were performed according to Laitinen et al. (8). For stability analysis, protein samples were combined with sample buffer and incubated at selected temperatures for 20 min before being subjected to SDS-PAGE (15). The quaternary status of avidin and the mutant was determined by fast-protein liquid chromatography, performed on a Superdex 200 HR 10/30 (Amersham Biosciences) using a Shimadzu SCL-10A VP system controller with RF-10A x1 fluorescence detector and SPD-M 10A VP diode array detector. Samples (sodium phosphate buffer, 0.65 M NaCl, pH 7.2) were applied, and chromatography was carried out at a flow rate of 0.5 ml/min.

ELISA Analyses—The immunological properties of the mutant were compared with avidin by an indirect ELISA analysis as previously described in Laitinen et al. (16). ELISAs were performed using two monoclonal avidin antibodies (produced at the Institute of Medical Technology, University of Tampere, Tampere, Finland) and a polyclonal rabbit avidin antibody (produced at the Laboratory Animal Center, University of Oulu, Oulu, Finland) as primary antibodies.

RESULTS

Construction, Production, and Purification of the Avidin Mutant—The W110K+N54A avidin mutant was produced in biotin-free insect cell medium as described previously (14). The mutant was purified efficiently on biotin agarose and eluted with mild acid treatment (8) to yield virtually pure mutant protein (not shown). The location of residues W110K and N54A in the tetramer structure is shown in Fig. 1. The tendency of the W110K substitution to form higher order quaternary structures (dimers, trimers, oligomers) was studied by SDS-PAGE (15.2 and 17.5 kDa, respectively), indicating a monomer in solution (Fig. 2A). The dissociation constant of mutant for the immobilized biotin was determined at 1–3 × 10⁻⁷ M in IAsys analyses (Fig. 2B, Table I). The binding and dissociation curves were consistent with simple first-order kinetics. The resultant kinetics data were in accord with self-consistency tests described by Schuck and Minton (17) whereby dissociation constants calculated from equilibrium response data were similar to those obtained directly from the binding curves (Table I). The binding was also examined under soluble conditions by fluorescence spectroscopy monitoring the quenching of the intrinsic fluorescence due to biotin binding to avidin. Total decrease of about 45% in protein intrinsic fluorescence intensity was observed when monomavirus sample was titrated with biotin (Fig. 2C). The fitted binding curve (r² = 0.98) gave dissociation constant of ~7.6 × 10⁻⁸ M.

Structural Analyses—The tendency of the W110K+N54A double mutant to form higher order quaternary structures (dimers, trimers, oligomers) was studied by SDS-PAGE based assay (15). The mutant migrated as a monomer even at room temperature, both in the absence and presence of biotin (not shown). The quaternary structural status of the mutant was also determined in solution by fast-protein liquid chromatography assay. The calculated value for the monomeric peptide sequence is 14.2 kDa (~15.7 kDa including the oligosaccharide side chain). This value fits well with the estimated molecular weights both in the absence or presence of biotin (15.2 and 17.5 kDa, respectively), indicating a monomer in solution in both cases. Biotin-free and biotin-bound forms of the
native protein were tetrameric (Fig. 3). Henceforth, the W110K+N54A mutant was termed monoavidin.

The resistance of monoavidin to proteolytic cleavage was studied by proteinase K treatment and compared with the wild-type avidin. In the absence of biotin, monoavidin was degraded within minutes, whereas the presence of the ligand had a short-lived protective effect on the mutant against the proteolytic action of proteinase K. Complete proteolysis took place within an hour, whereupon the protein was digested into small peptides. Native avidin was clearly more stable, both in the absence and especially in the presence of biotin (Fig. 4). As reported previously (18), the native protein was cleaved into two long peptides.

**Immunological Analyses**—Monoavidin was compared immunologically to native avidin by ELISA using a polyclonal and two monoclonal anti-avidin preparations. The polyclonal rabbit sera recognized monoavidin clearly weaker than native avidin and the monoclonal antibodies failed to recognize the mutant (Table II).

**DISCUSSION**

The linkage of the tetrameric architecture to the strong affinity of the avidin/streptavidin-biotin complex was first evident from biochemical studies and then confirmed by the determination of the crystal structures (3–6). In view of these findings, we and others have employed site-directed mutagenesis of avidin and/or streptavidin (8, 9, 12, 19–23), both to better understand this intimate structure-function relationship and to design mutated forms of the proteins that would exhibit interesting physicochemical or binding properties. In this context, it has been one of our expressed goals to prepare an active, reversible biotin-binding monomer of one or both proteins. Indeed, in recent work (8), we succeeded in producing a dimeric form of avidin and streptavidin that bound biotin reversibly. In another work, we developed monomeric forms of avidin that fortuitously assembled into tetramers upon binding biotin (12). The question outstanding was whether a combination of the two types of mutant would lead to a stable, reversible biotin-binding monomer.

**Table I**

| Affinity parameter | Value       |
|--------------------|-------------|
| $K_d$ (m)          | $1.4 \times 10^{-7}$ |
| $K_d$ (m)          | $2.9 \times 10^{-7}$ |
| $K_d$ (m)          | $1.1 \times 10^{-7}$ |
| $K_d$ (m)          | $2.0 \times 10^{4}$  |
| $K_d$ (m)          | $2.1 \times 10^{-3}$ |
| $K_d$ (m)          | $5.8 \times 10^{-3}$ |

$^a$ The dissociation constants were calculated from the equilibrium response data.

$^b$ Dissociation constant calculated from $k_a$ and $k_{diss}$ values, derived from association analysis using a plot of $k_a$ against protein concentration.

$^c$ The dissociation constant was calculated directly from the binding curves. The association and dissociation data were measured for 500 s in each concentration.

$^d$ $K_{diss}$ derived directly from dissociation curves.

$^e$ $k_{diss}$ derived from association analysis using a plot of $k_a$ against protein concentration.
The dimeric mutants of avidin and streptavidin were produced by rational design (8), which involved a radical point mutation of a single biotin-binding residue (i.e. Trp-110 in avidin or the conserved Trp-120 in streptavidin) to a lysine in both cases. The rationale for this particular “irrational” mutation was inspired by comparing the sequences of avidin and streptavidin to those of the sea urchin fibropellins (24). One of the domains of the latter proteins is remarkably similar to avidin and streptavidin, including most, but not all, of the biotin-binding residues. In this case, the designated binding site tryptophan in the avidins is replaced by a lysine in the fibropellins, hence our decision to effect the Trp→Lys mutations of avidin and streptavidin. Since the same tryptophan also plays a crucial role in the 1→2 monomer–monomer interface, this radical type of point mutation also generates dimeric forms of the two proteins.

The monomeric avidin mutants were also produced by rational design upon categorizing the molecular forces involved in maintaining the other two interfaces of the avidin tetramer.2 Using this approach, all three residues involved in the 1→3 interface were converted to alanines (12). The resultant 1→3 mutant still formed a tetramer, but its stability properties were compromised. In contrast to the relatively weak 1→3 intermonomer interaction, the 1→4 interface exhibits an extensive hydrogen bonding network. Nevertheless, one of the residues involved in this interface (Asn-54) contributes numerous hydrogen bonding interactions with several residues of the neighboring monomer. The combination of the single Asn-54→Ala mutation with those of the 1→3 interface was sufficient to disrupt the stable avidin tetramer into monomer (12). Such monomers, however, re-assembled into stable tetramers upon biotin binding.

In the present work, combined single point mutations of two critical 1→2 and 1→4 interface residues generated a stable avidin monomer. These data infer that once these two interfaces are disrupted, the relatively weak 1→3 interface is insufficient to maintain a viable avidin dimer. Disruption of the 1→2 and 1→4 intermonomer interactions apparently causes changes in the tertiary structure of the avidin monomer, since the monomer is more labile than the tetramer to protease K treatment. In the conditions where protease K cleaves wild-type avidin at only one position (18), monomeric avidin was completely digested into small peptides. Moreover, immunochemical analysis using polyclonal antiavidin antibodies revealed a reduction of cross-reactivity with monomeric avidin, and monoclonal antibodies (elicited against the native avidin tetramer) failed to react with the avidin monomer.

Monomeric avidin columns have been described in the past for isolation of biotynlated materials. The immobilized monomers were produced by treating native egg-white avidin columns with strong chemical denaturants (7, 25, 26). However, the avidin monomers tend to form tetramers spontaneously, despite the fact that the immobilized monomers are covalently bound to the resin (7). Once formed, the tetramers again bind biotin irreversibly. To preserve the reversibility of such columns, they are commonly stored in the presence of denaturant, but the long term utility of the column is thus compromised. The stable monomeric avidin described in the present communication provides a more effective and advantageous component for such columns. In addition, strategies for producing avidin-based fusion proteins would benefit from the use of monomeric avidin as a fusion partner, compared with using avidin tetramers. If a fusion partner (like avidin or streptavidin) has an intrinsic propensity toward oligomerization, the mature fusion protein would likely form higher order aggregates with undesirable physicochemical properties that would interfere with their intended function. It is not surprising that, to date, the most successful (strept)avidin-containing fusion proteins have included relatively small proteins as fusion partners, such as green fluorescent protein (27, 28) or antibody Fab fragments fused to streptavidin (29).

The reversible biotin-binding characteristics of monomeric avidin are also advantageous for isolation of monomeric avidin-containing fusion proteins. To date, the most popular affinity purification protocols for avidin have utilized 2-iminobiotin as an affinity matrix. The relevant protocols include a binding step under conditions of high pH (>10), whereas the elution step is accomplished by lowering the pH to 4 or even lower values (30). Many proteins, however, are labile at high or low pH values, and purification procedures that include such conditions may lead to inactive fusion products. Consequently, the reversible biotin-binding properties of monomeric avidin are amenable to protocols that require physiological binding conditions and mild elution with biotin.
Pérez-Luna et al. (31) have recently studied the wild-type and two tetrameric streptavidin mutants by surface plasmon resonance optical biosensor. They observed that dissociation rates for these streptavidin mutants did not follow the simple first-order kinetics in their experiment. The reasons for this behavior were proposed to be nonspecific interactions of the bound proteins for non-evenly organized surface, cooperativity of binding to a dense biotin surface caused by multiple binding sites in tetrameric proteins, and/or interactions between adsorbed proteins and tethering effects of the ligand. In contrast, first-order association and dissociation kinetics were obtained for monoaavidin using IAAs in the present study. Together with the complete reversibility of biotin binding, the data suggest that the latter phenomena did not significantly affect the observed interactions. Indeed, the lack of cooperativity would not be surprising due to the monovalency of the studied molecule. The character of the surface used for these experiments can also have a strong impact on the kinetic parameters. Edwards et al. (32) have compared the binding kinetics of human serum albumin to an antibody immobilized either to a dextran matrix or to an aminosilane surface. They found that when the dextran matrix was used the binding phenomena followed second-order kinetics due to steric hindrance between the binding matrix and the analyte, whereas kinetics obtained by using the aminosilane surface followed first-order kinetics. In this context, the binding analyses were performed on a planar aminosilane surface in the present study.

Kohanski and Lane (25) have previously described association and dissociation values for column-immobilized monomeric avidins (generated from egg white avidin by incubation with chaotropic agents) using radiolabeled biotin. The resultant monoaavidin is appropriate as a reversible monomers by changing only two structurally important amino acids. The resultant monoaavidin is appropriate as a reversible biotin-binding component of affinity columns and fusion proteins for use in developing novel applications of the (strept) avidin-biotin system.

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