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Dual-Affinity Avidin Molecules

Vesa P. Hytönen,[1#] Henri R. Nordlund,[1†] Jarno Hörhä,[1] Thomas K. M. Nyholm,[2] David E. Hyre,[3] Tuomas Kulomaa,[1] Eevaleena J. Porkka,[1] Ari T. Marttila,[1‡] Patrick S. Stayton,[3] Olli H. Laitinen,[4] and Markku S. Kulomaa[1†*]

[1]*NanoScience Center, Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland*
[2]*Department of Biochemistry and Pharmacy, Åbo Akademi University, Turku, Finland*
[3]*Department of Bioengineering, University of Washington, Seattle, Washington*
[4]*A. I. Virtanen Institute, Department of Molecular Medicine, University of Kuopio, Kuopio, Finland*

ABSTRACT **A recently reported dual-chain avidin was modified further to contain two distinct, independent types of ligand-binding sites within a single polypeptide chain. Chicken avidin is normally a tetrameric glycoprotein that binds water-soluble *d*-biotin with extreme affinity ($K_d \approx 10^{-15}$ *M*). Avidin is utilized in various applications and techniques in the life sciences and in the nanosciences. In a recent study, we described a novel avidin monomer-fusion chimera that joins two circularly permuted monomers into a single polypeptide chain. Two of these dual-chain avidins were observed to associate spontaneously to form a dimer equivalent to the wt tetramer. In the present study, we successfully used this scaffold to generate avidins in which the neighboring biotin-binding sites of dual-chain avidin exhibit two different affinities for biotin. In these novel avidins, one of the two binding sites in each polypeptide chain, the pseudodimer, is genetically modified to have lower binding affinity for biotin, whereas the remaining binding site still exhibits the high-affinity characteristic of the wt protein. The pseudotetramer (i.e., a dimer of dual-chain avidins) has two high and two lower affinity biotin-binding sites. The usefulness of these novel proteins was demonstrated by immobilizing dual-affinity avidin with its high-affinity sites. The sites with lower affinity were then used for affinity purification of a biotinylated enzyme. These "dual-affinity" avidin molecules open up wholly new possibilities in avidin–biotin technology, where they may have uses as novel bioseparation tools, carrier proteins, or nanoscale adapters. Proteins 2005;61:597–607.**

Key words: avidin; biotin; molecular engineering; mutagenesis; diagnostics; bioseparation; nanotechnology

INTRODUCTION

Avidin from chicken and its structural analogue, streptavidin, from the bacterium *Streptomyces avidinii*, are biotin-binding proteins exhibiting extraordinarily high affinity toward *d*-biotin, a small, water-soluble vitamin.[1] Avidin and streptavidin are antiparallel β-barrel proteins consisting of four identical subunits.[2–4] The quaternary structure assembly of the (strept)avidin tetramer positions two pairs of binding sites on opposite sides of the tetramer.[3,5]

Due to this tight and specific interaction, avidin and streptavidin have been exploited in various applications in the field of the life sciences.[6] Recently, avidin and streptavidin have found uses as building blocks in various nanotechnology studies, where they have acted as nanoscale connectors for biotinylated objects. For example, Hinds et al.[7] used biotin for carbon nanotube membrane functionalization. They used a biotin–streptavidin pair as a *nanovalve* to control the ion flux via the membrane. Keren et al.[8] employed streptavidin and a biotinylated antibody to generate a self-assembled nanotube *transistor*. In another study Banerjee et al.[9] functionalized the ends of peptide nanotubes with avidin and used these structures as self-assembling *nanowires* between biotin-functionalized gold electrodes. Since the functional and structural elements of (strept)avidin–biotin technology are well characterized, they can be modified in several ways so as to form suitable building blocks for use in nanotechnology. (Strept)avidin and biotin are also employed in various targeted drug delivery and gene therapy systems.[10–14]

Both avidin and streptavidin have been modified extensively for various purposes.[15–28] However, the possibilities of modification are limited by the homotetrameric assembly of (strept)avidin. It is not possible to modify only one

Abbreviations: Avd, chicken avidin protein; cDNA, complementary DNA; cpAvd5→4, circularly permuted avidin wherein the new N-terminus is before β-strand 5 and new C-terminus after β-strand 4; cpAvd6→5, circularly permuted avidin wherein the new N-terminus is before β-strand 6 and new C-terminus after β-strand 5; dcAvd, dual-chain avidin, wherein the circularly permuted avidins cpAvd5→4 and cpAvd6→5 are joined together in a single polypeptide chain; CSC, Calorimetry Sciences Corporation; DSC, differential scanning calorimeter; EMBL, European Molecular Biology Laboratory; FPLC, fast performance liquid chromatography; ITC, isothermal titration calorimeter; K_d, dissociation constant; k_{diss}, dissociation rate constant; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T_m, transition midpoint of heat denaturation; wt, wild-type.

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[#]Present address: Department of Materials, ETH Zürich, Hönggerberg, CH-8093, Zürich, Switzerland

[†]Present address: Institute of Medical Technology, FI-33014 University of Tampere, Tampere, Finland

[‡]Present address: Biofellows Oy, Koetilantie 1 C 18, FI-00790 Helsinki, Finland

*Correspondence to: Markku S. Kulomaa, Institute of Medical Technology, Biokatu 6, FI-33014 University of Tampere, Tampere, Finland. E-mail: markku.kulomaa@uta.fi

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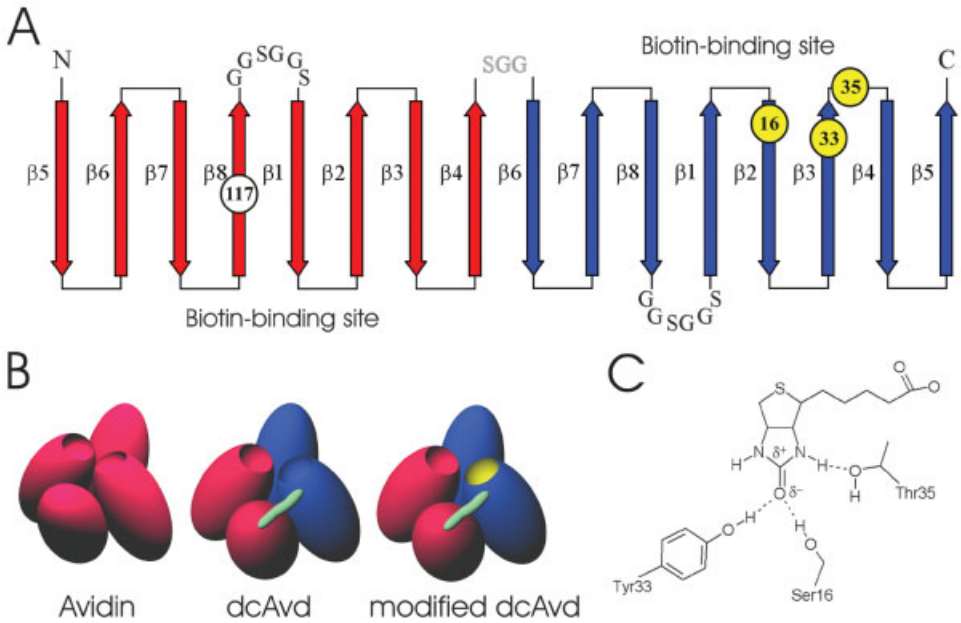

Fig. 1. Construction of dual-affinity avidins. (**A**) Representation of the topology of the secondary structure elements of dcAvd. The original circularly permuted monomers are colored red and blue. The peptide linkers connecting the old C- and N-termini of avidin in cpAvd5→4 (blue) and cpAvd6→5 (red), as well as the linker which connects the cpAvds to form dcAvd are shown in black and green letters, respectively. The locations of the mutagenized residues are shown by circles. The yellow circles refer to the modified biotin-binding residues. (**B**) Schematic representation of one possible quaternary structure of dcAvd and modified dcAvd. Color codes as in (A). The peptide linker connecting the cpAvds is shown as a green tube. (**C**) Schematic representation of the biotin-binding residues mutagenised in this study and their interactions with biotin.

subunit without making the same modification to all the other subunits. Avidin with nonidentical binding sites (i.e., a chimeric tetramer) would offer a new range of possibilities. For example, it would be useful to be able to release the bound ligand and a possible cargo in desired conditions from (strept)avidin[29–31] while still retaining other biotinylated molecules at the rest of the binding sites or using them to immobilize the (strept)avidin carrier by means of a suitable biotinylated matrix.

Hitherto, it has not been possible to combine different functions together in the same (strept)avidin molecule to increase the diversity of possible functions. One attempt to produce a chimeric streptavidin was presented by Chilkoti et al., who mixed two functionally different streptavidin monomers in a denatured state, then renatured them simultaneously.[32] This rather laborious procedure, however, did not generate homogenous material, but produced a binomial distribution of combinations. In order to overcome these and other limitations, we recently introduced a novel scaffold for avidin engineering.[33] In this dual-chain avidin, two different circularly permuted avidin monomers were genetically fused together (Fig. 1). This construct led to the formation of a polypeptide chain containing two distinct biotin-binding sites. Two of these polypeptide chains assemble spontaneously into a dimer, thereby creating a pseudotetrameric quaternary structure. In the present study we used this dual-chain scaffold to generate avidin pseudotetramers containing nonidentical binding sites. We constructed a set of dual-affinity avidin molecules in which two of the binding sites of the pseudotetramer have wild-type high affinity to biotin and two have lowered affinity. The new avidin constructs described in this study provide unique and versatile tools for use in avidin–biotin technology.

METHODS

Materials

Biotin was obtained from Sigma Chemicals. Chicken avidin, a generous gift from Belovo S. A. (Bastogne, Belgium) was used as a control protein in the analyses. The concentrations of the avidin solutions were calculated from the absorbance at 280 nm using an extinction coefficient of 24,280 M^{-1} cm^{-1} per binding site. Values of 24,340 M^{-1} cm^{-1}, 23,000 M^{-1} cm^{-1}, and 23,700 M^{-1} cm^{-1} were used for dcAvd($I117C_{5\rightarrow4}$), Avd(Y33H), and dcAvd($I117C_{5\rightarrow4}Y33H_{6\rightarrow5}$), respectively.

The sequence of dcAvd is available at EMBL by accession number AJ616762. We have used the amino acid numbering of wt avidin for mutations made to dcAvd construct throughout the present study (Table I). The corresponding residue changes numbered according to the mature dcAvd sequence are as follows: $I117C_{5\rightarrow4}$ = I62C, $S16A_{6\rightarrow5}$ = S216A, $Y33H_{6\rightarrow5}$ = Y233H and $T35A_{6\rightarrow5}$ = T235A (Fig. 1).

Design and Mutagenesis of the Expression Constructs and Production of the Recombinant Proteins

Site-directed mutagenesis of the cDNA encoding avidin, cpAvd6→5 or cpAvd5→4 was performed using the QuikChange (Stratagene, La Jolla, CA) or megaprimer[35] mutagenesis methods. Mutagenized forms of cpAvd6→5

TABLE I. Description of the Dual-Chain Avidin Mutants

Protein	Mutation in cpAvd5→4 domain	Mutation in cpAvd6→5 domain	Description	Reference
dcAvd	None	None	Original dual chain avidin, fusion of the two circularly permuted avidins	Nordlund et al.[33]
dcAvd($I117C_{5\rightarrow4}$)	I117C	None	Stabilizing disulfide bridge formation between the cpAvd5→4 domains of dcAvd	Reznik et al.[17]; Nordlund et al.[27]
dcAvd($I117C_{5\rightarrow4}S16A_{6\rightarrow5}$)	I117C	S16A	Mutation of a biotin-binding residue	Klumb et al.[34]
dcAvd($I117C_{5\rightarrow4}Y33H_{6\rightarrow5}$)	I117C	Y33H	Mutation of a biotin-binding residue	Marttila et al.[26]
dcAvd($I117C_{5\rightarrow4}T35A_{6\rightarrow5}$)	I117C	T35A	Mutation of a biotin-binding residue	Klumb et al.[34]

The mutated residues are numbered according to avidin polypeptide. References to the original publications describing the effects of particular mutations in avidin or streptavidin are also given.

and cpAvd5→4 were then joined together to form the chimeric dcAvds by ligation after BamHI/HindIII digestion into a pFASTBAC1 donor vector (Table I). Recombinant baculoviruses encoding different avidin forms in *Spodoptera frugiperda* Sf9 insect cells were generated using these donor vectors according to the Bac-To-Bac™ manufacturer's instructions (Gibco BRL, Life Technologies, Gaithersburg, MD). Proteins were produced in baculovirus-infected Sf9 insect cells in biotin-free medium as previously reported.[36] The produced proteins were purified in a single step by affinity chromatography on a 2-iminobiotin agarose column, as described elsewhere.[25]

Calorimetry

The biotin-binding thermodynamics of Avd(S16A) and Avd(T35A) were studied using a CSC ITC-4200 isothermal titration calorimeter. The measurements were performed at 25°C and 37°C, as previously described.[37] The T_m of heat denaturation of the various avidins was studied using a CSC Nano II DSC as previously described.[27,38] A 50 m*M* Na-PO_4 buffer with 100 m*M* NaCl (pH 7.0) was used in both the ITC and DSC measurements.

Calculation of Binding Constants

Binding constants were calculated from a combination of ITC and DSC data according to Brandts and Lin.[39] The method is based on the differences in the T_m of denaturation in the presence and absence of the ligand. The ΔC_p value 15.3 kJ/K mol determined previously for avidin was used in the calculations.[37]

Interaction Analysis

The 2-iminobiotin-binding characteristics of the different avidins were studied using an IAsys optical biosensor. Measurement was carried out using a 2-iminobiotin surface in 50 m*M* borate buffer (pH 9.5, 1 *M* NaCl). The binding equilibrium was measured in various protein concentrations, and the binding curve was fitted to the resulting data to determine the apparent K_d, as previously reported.[25]

Radiobiotin Dissociation Assay

The dissociation rate constant of [^{3}H]biotin from avidin, dcAvd, and the circularly permuted avidins cpAvd5→4 and cpAvd6→5 was determined by competition with free biotin at three temperatures as described in Klumb et al.[34]

Dissociation Analysis by Fluorescent Biotin

The biotin-binding properties of the avidins were analyzed by measuring the quenching, due to binding of avidin, of a ArcDia™ BF560 biotin-coupled fluorescent probe (ArcDia Ltd., Turku, Finland).[40,41] A PerkinElmer LS55 luminometer was used in these measurements, which were carried out in 50 m*M* Na-phosphate, pH 7.0, containing 650 m*M* NaCl, at room temperature (23 ± 1°C), as previously described.[41]

A one-phase dissociation model was used to analyze the data, as described elsewhere.[41] The dissociation rate constant (k_{diss}) was determined by fitting the equation $-k_{diss}t = \ln(B/B_o)$ to the data. B_o is the measured maximum binding (100%) determined as the difference between the fluorescence of the free dye and the fluorescence of the protein–dye complex, and B is the amount of complex measured as a function of time. The first 500 s were omitted from the data to abolish the effect of the fast initial-phase characteristic of avidin–BF560–biotin interaction (Fig. 2). The fraction of fluorescent biotin released after 1 h was determined, as well as the times of half-maximal ($t^1_{\frac{1}{2}}$) and quarter-maximal ($t^1_{\frac{1}{4}}$) release. For dual-chain avidins with modified binding sites, it was hypothesized that 50% of the binding sites would behave like those of wt avidin (cpAvd5→4 binding site of dcAvd was not modified). The dissociation data measured for wt avidin divided by two was subtracted from the data measured for the modified dual-chain avidins, and the dissociation curve was fitted to the difference as above.

Gel Filtration Analysis

The oligomeric state of the proteins was assayed with FPLC gel filtration at room temperature, as previously described.[42] Na-phosphate buffer (50 m*M*, pH 7.0) with 650 m*M* NaCl was used as the liquid phase. The high concentration of NaCl reduces the stacking of the basic avidin ($pI \approx 10.5$)[1] in the chromatography column.

SDS-PAGE-Based Thermostability Assay

Protein samples were acetylated in vitro, and temperature-dependent dissociation of the subunits after heat

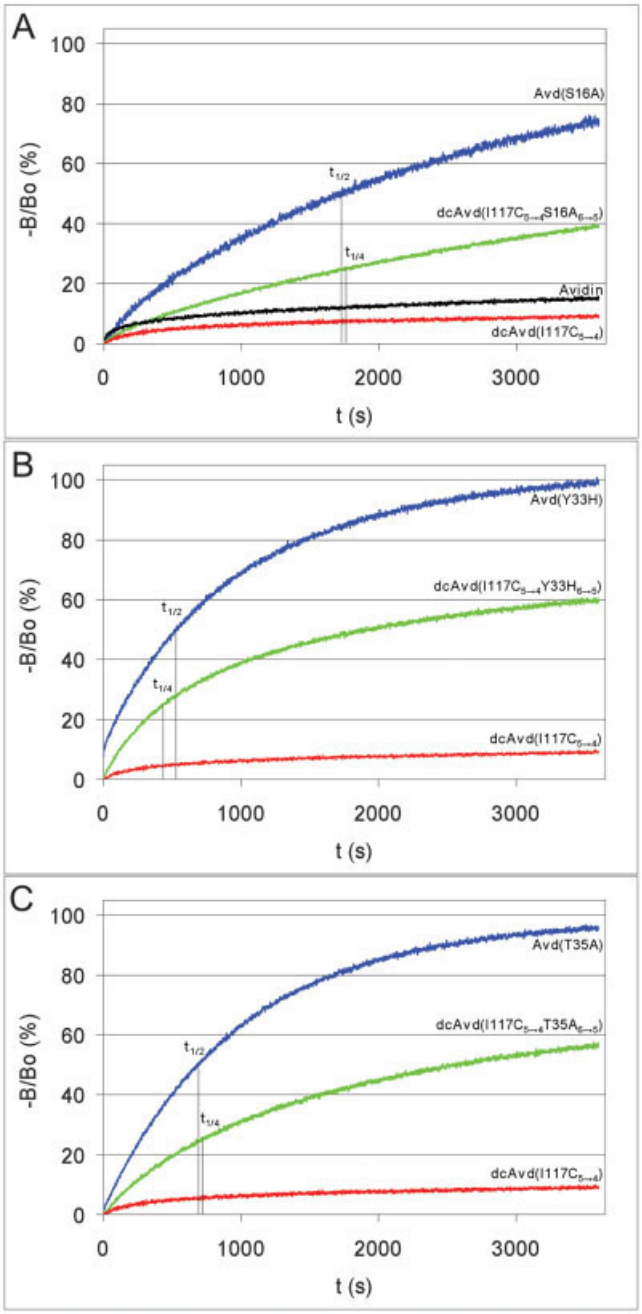

Fig. 2. The dissociation assay with fluorescent biotin conjugate. The protein under study was allowed to bind BF560–biotin conjugate, which causes fluorescence quenching. After that, a 100-fold excess of free biotin was applied to the sample and the release of the fluorescent biotin was observed via the increase in the fluorescence signal. The time points determined for 25% release ($t_{\frac{1}{4}}$) of the fluorescent biotin conjugate for dcAvd samples and 50% release ($t_{\frac{1}{2}}$) of conjugate for mutated avidins are indicated.

treatment for 20 min at given temperature between 25°C and 100°C was monitored from Coomassie-stained SDS-PAGE gels as described in detail by Bayer et al.[43]

Demonstration of Feasibility

The *d*-biotin Sepharose® 4 FF (Affiland S.A., Ans-Liege, Belgium) column (100 μL suspension) was allowed to react with 0.3 mg dcAvd(I117$C_{5\rightarrow4}$Y33$H_{6\rightarrow5}$) in 50 m*M* Na-acetate, pH 4.0, containing 100 m*M* NaCl. An equal amount of chicken avidin (Belovo S. A., Bastogne, Belgium) was added to a control column. The column was washed with 50 m*M* Na-carbonate, pH 11.0, containing 1 *M* NaCl. Biotinylated calf intestine alkaline phosphatase (5.5 μg; Pierce, Rockford, IL) was then added to the column, and it was again washed with 50 m*M* Na-carbonate, pH 11.0, containing 1 *M* NaCl. Elution was performed by 50 m*M* Na-acetate, pH 4.0, containing 100 m*M* NaCl. The enzyme activity in fractions was measured by diluting a small sample to 1 *M* diethylamine, pH 9.6, containing 1 mg/mL *p*-nitrophenyl phosphate, and the absorbance was measured at 405 nm after 40 min incubation. Another cycle of binding/eluting was done with the same columns after washing with 50 m*M* Na-carbonate, pH 11.0, containing 1 *M* NaCl.

RESULTS

Protein Production and Purification

The produced proteins (Table I) were purified efficiently by 2-iminobiotin affinity chromatography, which indicated that they were all soluble and biologically active in the sense of ligand binding.

In order to study the quaternary structure of the produced avidins, they were analyzed using FPLC gel filtration assay. All the proteins eluted mainly at the same volume as wt avidin, roughly 55 kDa; that is, they were tetramers (avidin mutants and cpAvds) or dimers (dcAvd and its variants) in size (Table II).

Biotin-Binding Thermodynamics

Thermodynamics of biotin binding to the proteins was examined using ITC. The results are shown in Table III. Because Avd(S16A) and Avd(T35A) had very high affinity for biotin, we could not calculate the binding constants directly from the ITC experiments. However, since, in principle, all the ligand is bound in every titration, the binding enthalpy could be calculated directly from the heat released upon the injection of biotin into the sample cell. Wild-type avidin had the highest binding enthalpy of these three proteins at 25°C. The ΔC_{pL} in the wt avidin and Avd(T35A) was similar, but the Avd(S16A) had a more negative ΔC_{pL}, indicating more temperature-dependent binding behavior as compared to the other proteins.

Biotin-Binding Analyses

The biotin-binding properties of the decreased affinity avidin mutants S16A and T35A were studied with DSC and ITC analyses, optical biosensor analysis, and fluorescent biotin conjugate assay. Calorimetric analyses showed a 13-fold and 238-fold decrease in the biotin-binding affinity for Avd(S16A) and Avd(T35A), respectively, at 25°C when compared to that of wt avidin (Table III). Similarly, the mutants showed lowered affinity to 2-iminobiotin surface when compared to that of wt avidin (Table III). The assay with fluorescent biotin revealed increased dissociation rate constants (k_{diss}) for these mutants when compared to that of avidin (Table IV), reflecting lower

TABLE II. Stability of the Proteins

Protein	SDS-PAGE stability assay T_t/°C (−)biotin	SDS-PAGE stability assay T_t/°C (+)biotin	DSC T_m/°C (−)biotin	DSC T_m/°C (+)biotin	DSC ΔT_m[a] (°C)	Gel filtration MW (kDa)
Avidin	60	95	83.5 ± 0.1	117.0 ± 0.7	33.5	58.4
Avd(S16A)	55	80	84.3 ± 0.3	114.6 ± 0.2	30.3	55.9
Avd(Y33H)	ND[b]	75	73.0	106.3	33.3	51.0
Avd(T35A)	55	80	82.6 ± 0.1	113.2 ± 0.1	30.6	55.3
cpAvd5→4	45[c]	75[c]	72.7 ± 0.4	111.3 ± 1.1	38.6	45.9
cpAvd6→5	40[c]	70[c]	65.6 ± 0.7	102.0	36.4	48.0
dcAvd	40[c]	75[c]	80.2 ± 0.0	115.9	35.7	40.7
dcAvd(I117C$_{5\rightarrow4}$)	60	85	87.7 ± 0.1	118.0	30.3	43.0
dcAvd(I117C$_{5\rightarrow4}$S16A$_{6\rightarrow5}$)	60	80	87.7	112.9	25.2	51.3
dcAvd(I117C$_{5\rightarrow4}$Y33H$_{6\rightarrow5}$)	ND[b]	65	81.6	106.2	24.6	53.4
dcAvd(I117C$_{5\rightarrow4}$T35A$_{6\rightarrow5}$)	60	75	89.6	113.0	23.4	55.3

Transition midpoint temperatures of the oligomeric disassembly measured by an SDS-PAGE-based method. Heat-induced unfolding of the proteins determined by DSC. Apparent molecular weight obtained by gel filtration analysis.
[a] ΔT_m is the change in T_m upon addition of a three-fold molar excess of biotin.
[b] More than 50% of protein appears in the monomeric form in SDS-PAGE already at room temperature.
[c] From Nordlund et al.[33]

TABLE III. Biotin-Binding Analyses of the Decreased-Affinity Avidin Mutants

	K_b[a] (25°C) (M^{-1})	K_d[a] (25°C) (M)	ΔH (25°C) (kJ/mol)	ΔH (37°C) (kJ/mol)	ΔC_{PL} (kJ/K mol)	ΔG (25°C) (kJ/mol)	ΔS (25°C) (J/K mol)	K_d (2-iminobiotin, 20°C)[c] (M)
Avidin[b]	9.3×10^{15}	1.1×10^{-16}	−109.3 ± 4.0	−130.4 ± 5.5	−1.76	−91.2	−60.8	2.2×10^{-8}
Avd(S16A)	7.3×10^{14}	1.4×10^{-15}	−96.28 ± 3.6	−137.4 ± 2.9	−3.43	−84.9	−38.3	9.7×10^{-8}
Avd(T35A)	3.9×10^{13}	2.6×10^{-14}	−75.0 ± 2.8	−99.7 ± 0.3	−2.06	−77.6	−87.3	1.1×10^{-6}

Thermodynamic parameters for the binding of biotin to wt and mutant avidins. All values are averages from two different experiments ± standard deviation. The ΔG is calculated from the equation $\Delta G = -RT \ln K_b$ and the ΔS is obtained from the relation $\Delta S = (\Delta H - \Delta G)/T$. Affinit y to 2-iminobiotin surface measured at 20°C by an IAsys optical biosensor is also indicated.
[a] The binding constant K_b calculated from the DSC and ITC results as described elsewhere.[37,39]
[b] From Hytönen et al.[37]
[c] Dissociation constant determined on basis of the binding equilibrium measured in various protein concentrations.

affinity to the conjugated biotin. The increase was 16-fold in the case of Avd(S16A) and 38-fold in the case of Avd(T35A).

Avidin mutant Y33H has previously been described as having lowered affinity to the 2-iminobiotin surface ($K_d \approx 1.2 \times 10^{-7}$ M) when compared to that of wt avidin ($K_d \approx 2.2 \times 10^{-8}$ M).[26] We observed a 48-fold increase in the dissociation rate constant in the assay with fluorescent biotin for this mutant when compared to that of avidin (Table IV).

Affinities to the 2-iminobiotin surface (K_d) for cpAvd5→4, cpAvd6→5, and dcAvd were 1.5×10^{-8} M, 5.5×10^{-7} M, and 4.4×10^{-8} M, respectively.[33] In order to further understand the biotin-binding properties of these proteins, a [^{3}H]biotin-dissociation assay was done for them. In this analysis, dcAvd showed tight wt avidinlike biotin binding, whereas cpAvd5→4 and especially cpAvd6→5, showed faster dissociation rates (Fig. 3). The fluorescent biotin assay results (Table IV) are in line with these analyses.

In order to study whether the new mixed-affinity dcAvd-proteins behaved as expected in the sense of biotin binding, they were also analyzed by the fluorescent biotin assay. In this analysis, dcAvd showed tight avidinlike biotin-binding (Table IV). On the basis of the results, the introduction of a stabilizing disulfide bridge between subunits had no effect on the biotin binding, since dcAvd(I117C$_{5\rightarrow4}$) showed binding characteristics similar to those of dcAvd (Table IV). When mutagenesis was used to alter the biotin-binding residues in two selected binding sites of dcAvd, a significant effect was observed. In the case of Y33H and T35A-mutations, about half of the bound biotin was released during the 1 h measurement (Fig. 2). The mutation S16A also caused a faster dissociation rate (Fig. 2, Table IV). Since only two out of the four binding sites in these pseudotetrameric avidins were modified, they seemed to behave as independent combinations of wt and mutagenized avidins (Table IV).

A two-independent-site model was generated to describe the biotin-binding properties of the modified dual-chain molecules, in which half of the binding sites behave as determined experimentally for unmodified binding sites (wt avidin) and half were considered as modified sites. The rationale for this assumption is that the cpAvd5→4 part of the dcAvd does not have modifications close the biotin-binding site (Fig. 1). Furthermore, the experimental analysis of the dcAvd biotin-binding properties showed that all the binding sites of dcAvd have high avidinlike biotin-

TABLE IV. Dissociation Assay With Fluorescent Biotin Conjugate

Protein	Release 1 h (%)	$t_{1/4}$ (s)[a]	$t_{1/2}$ (s)[a]	k_{diss} (s^{-1})
Avidin	14.1	ND[b]	ND	2.3×10^{-5}
Avd(S16A)	74.5	621	1728	3.6×10^{-4}
Avd(Y33H)	99.2	149	530	1.1×10^{-3}
Avd(T35A)	95.8	270	693	8.6×10^{-4}
cpAvd5→4	20.6	ND	ND	3.4×10^{-5}
cpAvd6→5	25.3	3557	ND	3.9×10^{-5}
dcAvd	10.1	ND	ND	1.4×10^{-5}
dcAvd($I117C_{5\rightarrow4}$)	9.1	ND	ND	1.3×10^{-5}
dcAvd($I117C_{5\rightarrow4}S16A_{6\rightarrow5}$)	39.1	1762	ND	2.8×10^{-4c}
dcAvd($I117C_{5\rightarrow4}Y33H_{6\rightarrow5}$)	59.9	435	1918	1.2×10^{-3c}
dcAvd($I117C_{5\rightarrow4}T35A_{6\rightarrow5}$)	56.9	723	2585	7.8×10^{-4c}

Dissociation of BF560–biotin conjugate from the proteins was measured at 23 ± 1°C by a luminescence spectrometer. Release of the fluorescent biotin conjugate competed with 100-fold molar excess of free biotin.
[a]The time point of 25% and 50% release was determined directly from the measured data.
[b]Not applicable in the case of a 1 h measurement.
[c]Dissociation rate for mutated binding site after subtraction of wt data. Observed data on wt avidin were used in a model to calculate the dissociation rate constant for the modified binding sites. In this model, it was hypothesized that 50% of the binding sites behave like the wt protein. Dissociation rates for the mutated binding sites were obtained by fitting the dissociation curve to the remaining 50% of sites by using the sum of the difference.

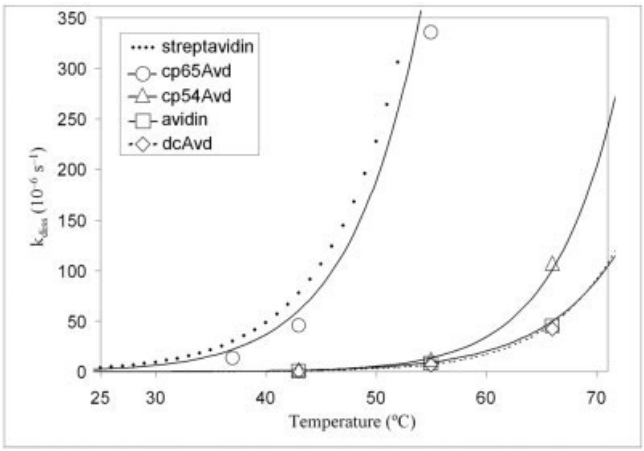

Fig. 3. Temperature-dependence of biotin dissociation rates measured by the [^{3}H]biotin dissociation assay. Dissociation rate constant of radiobiotin was determined at various temperatures and are shown by symbols. The global fit model[44] for dissociation rate constant obtained from combination of data from all experiments is shown by line (dcAvd: dotted line). The dissociation rate global model determined previously for streptavidin dissociation is also shown by black dots.[34]

binding affinity (Table IV, Fig. 3), which was also observed in the previous study.[33] The data measured for wt avidin were therefore divided by two and then subtracted from the measured dissociation data of the modified dcAvd-molecules to give the apparent behavior of the two mutagenized binding sites in the pseudotetramer. A single-phase dissociation curve was fitted to the remaining data. The dissociation rate values obtained by this model were close to those measured for analogous avidin mutants (Table IV). To validate our model, we also compared the values determined directly from the data for $t_{\frac{1}{2}}$ and $t_{\frac{1}{4}}$. The values of $t_{\frac{1}{2}}$ obtained for the avidin mutants were fairly similar to the $t_{\frac{1}{4}}$ values obtained for dcAvd-proteins containing the analogous mutations in half of the binding sites (Table IV, Fig. 2) as expected on the basis of the model. In other words, the $t_{\frac{1}{4}}$ values obtained for dcAvd mutagenized at one of its two sites give the $t_{\frac{1}{2}}$ values for the fast releasing sites when wt sites are excluded from the analysis by subtraction.

Stability of the Proteins

The role of the applied mutations to the thermal stability of avidin and dcAvd scaffold was studied by the examination the unfolding of the mutant avidins using DSC. The results are summarized in Table II. Both Avd(T35A) and Avd(S16A) unfolded at temperatures similar to wt avidin, whereas Avd(Y33H) showed lower stability. CpAvd5→4 and cpAvd6→5 showed lower melting temperatures than wt avidin. However, dcAvd showed almost identical thermal stability to that of wt avidin. With the addition of biotin (3:1 ratio; biotin:binding site) the T_m was shifted to a clearly higher temperature in all proteins. The obtained ΔT_m reflects also the biotin-binding affinity of a particular protein, although ΔT_m values of proteins showing similar stability in the absence of ligand should only be compared directly to each other. For example, avidin and Avd(S16A) apoforms showed stability similar to each other, but the mutant had smaller increase in T_m in the presence of biotin.

The stability of the modified dcAvd molecules was also determined by DSC. An increase in T_m was observed when mutation I117C was applied to dcAvd (Table II). When mutations S16A and T35A were applied to dcAvd($I117C_{5\rightarrow4}$), only relatively small changes in T_m, similar to those in wt avidin, were observed. The addition of the Y33H mutation, however, caused a more significant decrease in the T_m of dcAvd($I117C_{5\rightarrow4}$). Typical DSC scan profiles are shown in Figure 4.

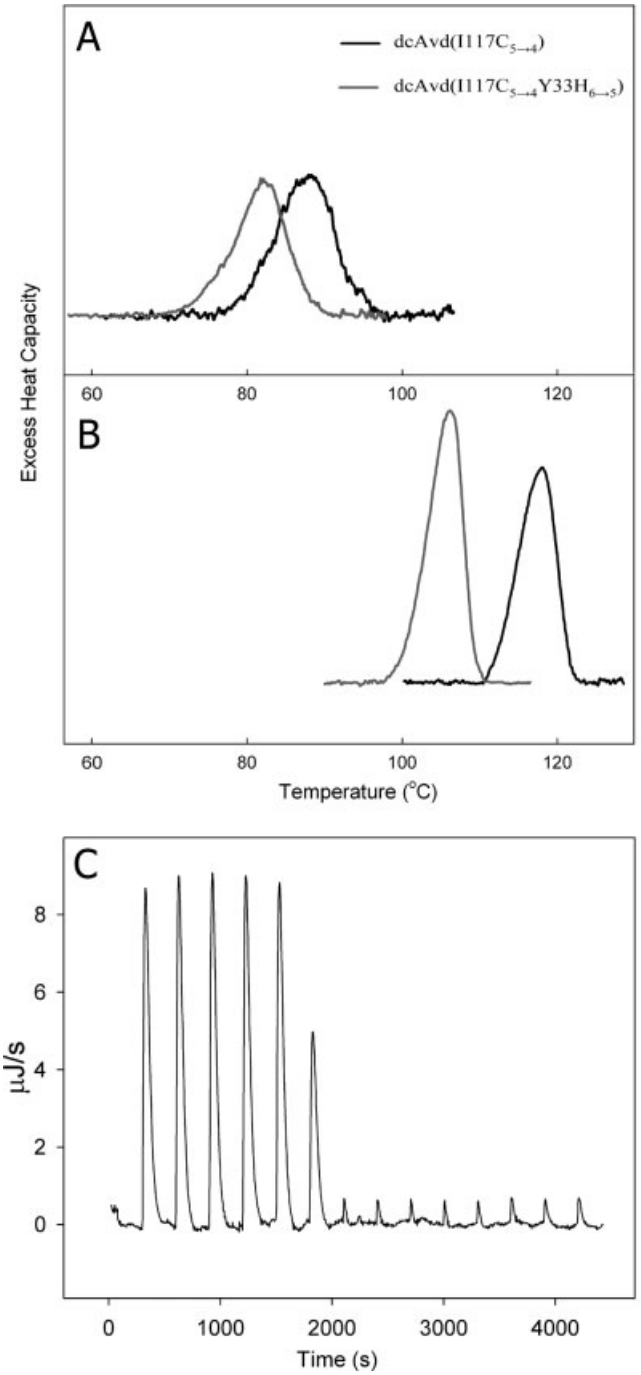

Fig. 4. Calorimetric analyses. (**A**) Thermograms from DSC analysis of dcAvd(I117C$_{5\rightarrow4}$) and dcAvd(I117C$_{5\rightarrow4}$Y33H$_{6\rightarrow5}$). (**B**) Thermograms from DSC analysis of dcAvd(I117C$_{5\rightarrow4}$) and dcAvd(I117C$_{5\rightarrow4}$Y33H$_{6\rightarrow5}$) in the presence of biotin. The addition of biotin clearly increases the transition midpoint of thermal denaturation (T_m). The effect of the mutation Y33H applied to half of the biotin-binding sites of dcAvd is seen as a decrease in the melting temperature both in absence and presence of biotin. (**C**) Representative ITC analysis. Titration of biotin into a sample of avidin mutant S16A at 25°C. There is sharp decrease in the released heat after sixth addition of biotin, indicating tight ligand binding.

The alternative SDS-PAGE based stability assay[43] gave results similar to DSC (Table II). Mutations S16A and T35A did not have a significant effect on the stability of

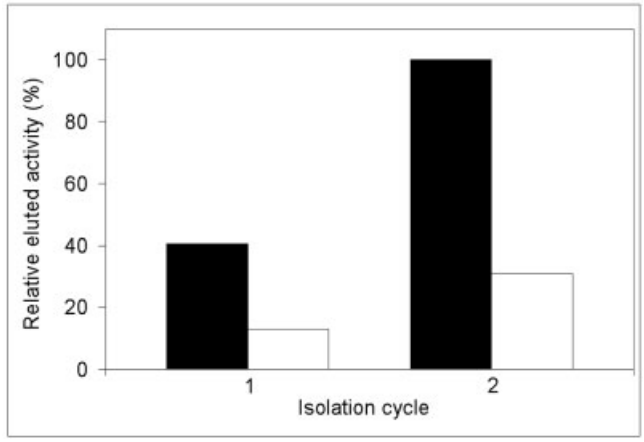

Fig. 5. Demonstration of feasibility. The affinity column was prepared by mixing biotinylated affinity matrix and dcAvd(I117C$_{5\rightarrow4}$Y33H$_{6\rightarrow5}$) protein. Biotinylated alkaline phosphatase was eluted from column by change in pH. The isolation efficiency was significantly higher (black) in two sequential purification cycles when compared to wt avidin (white). Another sequential isolation cycle was also performed to prove the stability of the column.

avidins. Mutation Y33H, however, radically destabilized the avidin in this assay.[26] The introduction of mutation I117C$_{5\rightarrow4}$ to dcAvd stabilized the protein in a manner similar to that previously described for wt avidin.[27]

Feasibility of Dual-Affinity Avidins in Applications

The usefulness of the dcAvd(I117C$_{5\rightarrow4}$Y33H$_{6\rightarrow5}$) for chromatographic isolation was proved in affinity purification experiment of biotinylated alkaline phosphatase. The affinity column was prepared by mixing biotinylated affinity matrix and dcAvd(I117C$_{5\rightarrow4}$Y33H$_{6\rightarrow5}$) protein. Biotinylated alkaline phosphatase was eluted from the column by change in pH. The isolation efficiency was significantly higher in two sequential purification cycles when compared to wt avidin. The efficiency of the column was increased 2.5-fold after the first isolation cycle. The results indicate that dcAvd(I117C$_{5\rightarrow4}$Y33H$_{6\rightarrow5}$) can easily be immobilized to a biotinylated matrix and then used to bind and release a biotinylated molecule more efficiently than wt avidin (Fig. 5).

DISCUSSION

Many applications of avidin–biotin technology would benefit from availability of avidins whose neighboring biotin-binding sites have different affinities for the ligand. Moreover, completely new applications could be designed on the basis of such dual-affinity avidins, where nonidentical binding sites permit the controlled release of biotinylated molecules in desired conditions. We recently introduced a novel avidin scaffold, dual-chain avidin or dcAvd, in which the subunits were circularly permuted and genetically fused together to form a pseudodimer.[33] Two dcAvds were found to form a dimer with four biotin-binding sites, thus forming a pseudotetramer. The existence of this new protein scaffold made it possible to independently alter the ligand-binding properties at half

of the binding sites. In the present study we utilized this scaffold, gradually lowering the biotin-binding affinity in half of the binding sites, while the remaining two were preserved as wt-like high-affinity sites. In order to lower the biotin-binding affinity of a binding site in dual-chain avidin, we first created and characterized two new point-mutated avidins, Avd(S16A) and Avd(T35A). As a third mutant in the reduced affinity set, we used the previously described Y33H mutation,[26] which is known to affect the biotin-binding properties of avidin. These three mutations were then individually applied to only one of the two binding sites [i.e., cpAvd6→5 part, of the dcAvd scaffold (Fig. 1)].

Effects of the Binding-Site Mutations on the Properties of Avidin

By mutating S16 and T35 separately to alanine in avidin, we obtained, according to the calorimetric analyses, 13-fold and 238-fold decrease, respectively, in their biotin-binding affinities. The increased dissociation rate was found mainly to explain the lower affinity of the mutated proteins as compared to wt avidin. The dissociation rate constant for avidin–biotin interaction has been reported to be $0.4–0.75 \times 10^{-7}$ s^{-1} (pH 7, 25°C).[45,46] However, it is known that, in some cases, the covalent attachment of various molecules to biotin increases its dissociation rate.[47] Accordingly, the present dissociation rates, determined by the fluorescent biotin method, were influenced by this phenomenon due to the conjugated fluorescent label. However, this is also the case in most of the applications of avidin–biotin methodology, and therefore, the values obtained are relevant to a number of applications. In addition, the dissociation rates determined by the radiobiotin assay for wt avidin gave, at ambient temperatures, values resembling those reported previously.[45,46]

Klumb et al.[34] measured a 161-fold decrease in the binding affinity of the S27A mutant of streptavidin, as compared to that of wt streptavidin. Our analogous S16A mutant had only a 13-fold decrease in affinity when compared to wt avidin. In our previous study, alanine mutation in S45 in streptavidin, which is functionally analogous to T35 in avidin, caused a 907-fold decrease in binding affinity[44] compared to the 238-fold decrease in binding affinity, due to mutation T35A in avidin, observed in the present study. The apparent differences in the changes in affinity between the analogous avidin and streptavidin mutants may be explained by the structural differences between these proteins, or perhaps by the very different methods of determining affinities in each case. A previous study has revealed a difference between the roles of T35 and S45 when biotin was conjugated to *p*-nitrophenyl anilide.[48] T35 in avidin was found to lose the hydrogen bond to biotin due to the conjugation of biotin to the *p*-nitrophenyl anilide, whereas in streptavidin the S45-biotin hydrogen bond continued to be present in the complex.[48] In another study, Y43 of streptavidin (analogous to Y33 in avidin) also mutated into phenylalanine and alanine, and five-fold and 93-fold increased dissociation rates, respectively, were observed.[34] Accordingly, we have previously changed Y33 to phenylalanine and histidine in avidin and observed a respective 13-fold and 22-fold increase in its 2-iminobiotin dissociation rate.[26] The mutation of Y33H in avidin increased the fluorescent biotin dissociation rate 48-fold.

The observed decrease in free energy of ligand binding due to mutations S16A and T35A ($\Delta\Delta G$ = 6.3 kJ/mol and 13.6 kJ/mol, respectively) is in line with that obtained previously when hydrogen-bonding side-chains with one charged partner were mutated.[49] This suggests binding mode for biotin in ureido–oxyanion resonance form in avidin similar to hypothesized for streptavidin by Weber et al.[50] Furthermore, similar decrease in binding energy have previously been measured for analogous streptavidin mutants S27A and S45A ($\Delta\Delta G$ = 12.1 kJ/mol and 17.6 kJ/mol, respectively).[34,44] The thermodynamic analysis of avidin mutant S16A showed only negligible decrease in the binding enthalpy (ΔH) compared to wt. The change was more significant in the case of mutant T35A (Table III). In addition to changes in bonding energy between protein and ligand, large changes in binding enthalpy could also reflect changes in ligand-binding mode or in protein conformation. Mutation S45A in streptavidin (position analogous to T35 in avidin) has previously been found to cause a significant change in the binding enthalpy compared to wt protein ($\Delta\Delta H$ = 31.4 kJ/mol, 37°C).[44] However, structural analysis of streptavidin S45A mutant revealed only negligible changes in the protein conformation. Therefore, the observed change in the binding enthalpy of Avd(T35A) compared to wt ($\Delta\Delta H$ = 30.7 kJ/mol, 37°C) might be associated with the absence of the hydrogen bond between biotin and mutated residue 35. However, avidin and streptavidin have differences in the structure of loop between β3 and β4, and therefore, the straightforward comparison of the analogous mutants does not fully prove this assumption.

The change in heat capacity upon ligand binding (ΔC_{PL}) is a rough measure of the surface area buried in the ligand-binding reaction. It is related to changes in hydration of protein or changes in assembly of solvent molecules at the protein surface. In the case of both mutated proteins Avd(S16A) and Avd(T35A), the measured ΔC_{PL} was larger compared to that of wt avidin (Table III). The more negative ΔC_{PL} could indicate that more apolar protein surface is buried in binding process. This might be at least partially explained by the more hydrophobic nature of the alanine residue compared to serine and threonine, since both these positions are in contact with solvent before ligand binding and became buried during the binding process.[3] Since serine is a more hydrophilic residue than threonine, the mutagenesis changes the nature of the binding site more apolar in the case of mutation S16A compared to mutation T35A. This could explain the larger ΔC_{PL} observed for Avd(S16A) compared to Avd(T35A). However, the mutations are in different positions, and therefore, are not directly comparable to each other. The observed effect can also arise from slight structural changes in protein caused by the mutagenesis.

Properties of Circularly Permuted Avidins, dcAvd and dcAvd(I117C$_{5\rightarrow4}$)

We observed in a recent study[33] that the components of dcAvd (i.e., cpAvd$_{5\rightarrow4}$ and especially cpAvd$_{6\rightarrow5}$) display somewhat weaker biotin-binding characteristics when compared to those of wt avidin. However, according to the radiobiotin dissociation assay, the ligand-binding affinity even of cpAvd6→5 was comparable to that of streptavidin (Fig. 3). Furthermore, the analyses used in the current study indicated that the combination of these two circularly permuted avidins in dcAvd had affinity toward biotin comparable with wt avidin. One possible reason for the difference might be the better stability of the dcAvd pseudotetramer when compared to the tetramers of the cpAvd's (Table II). The gain in entropy when the two chains of a dcAvd dimer (i.e., pseudotetramer) dissociate and unfold is clearly lower than that when the four chains of a wt or cpAvd homotetramer dissociate and unfold, effectively stabilizing the dcAvd because of cratic effects. Furthermore, the fixation of the N-terminus of cpAvd6→5 in dcAvd might also have an effect on ligand binding, since residues S75 and T77 close to the N-terminus of cpAvd6→5[33] form direct contacts with biotin.[3]

We found previously that dcAvd was thermally less stable than wt avidin.[33] In order to improve the stability of dcAvd, an I117C mutation was introduced into its cpAvd5→4-derived part. This modification should form one intermolecular disulphide bridge between the dcAvd subunits,[27] and therefore, fix their relative orientation at the level of the quaternary structure. The thermal stability of the dcAvd mutant was found to be comparable to that of wt avidin. Moreover, the biotin-binding properties of the dcAvd(I117C$_{5\rightarrow4}$) were as good as those prior to modification. Together, these observed properties validated it as a suitable precursor protein. Half of its binding sites (cpAvd6→5 derived domains) were made quickly reversible by applying the above-described point mutations to the dcAvd(I117C$_{5\rightarrow4}$) scaffold.

Characterization of Dual-Affinity Avidins and Their Applicable Potential

All of the produced dcAvd proteins with their affinity-decreasing mutations showed the desired properties. The binding sites containing mutations T35A and Y33H released biotin within 1 h, whereas the unmodified binding sites had binding ability resembling that of wt avidin. Similar but milder behavior was also observed with dcAvd containing mutation S16A. The changes in biotin-binding properties are also reflected in the thermal stability in the presence of biotin. The denaturation midpoint temperature (T_m) of holoprotein is lower in the case of modified dcAvds as compared to that of dcAvd with unmodified binding sites (Table II). Altogether, the dcAvd forms were found to have high thermal stability even after mutagenesis of biotin-binding residues, in contrast to the separate component binding-site mutants and circularly permuted avidins. These results suggest that even more radical manipulation could be performed on half of the binding sites in dcAvd without disturbing the high affinity biotin binding in the wt-like binding sites, such as mutagenesis of the residue W110[15,22,51] or multiple simultaneous mutations.

The dual-affinity avidins described in this study can be utilized in numerous different bioseparation techniques.[52] For example, these molecules allow the dcAvd mutant to be firmly immobilized on a biotin-functionalized surface or on a carrier material by the high-affinity native binding sites, while enabling use of the modified free binding sites with selected affinity for the catch and release of biotin-coupled molecules. This option was also experimentally demonstrated in this study (Fig. 5). In this sense, dual-affinity avidins immobilized to biotinylated beads could be used instead of streptavidin coated beads allowing release of the captured target molecule in mild conditions.[53] Likewise, the above-described avidins might find use in nanotechnology (e.g., in generation of more sophisticated structures and materials). The two high-affinity binding sites may be used for attachment of the protein via biotin to other building blocks in the molecular setting, and the rapidly reversible sites could be used as dynamic binding sites controlled, for example, by the concentration of free ligands. The dynamic binding site could function as a switch between two states controlled by the conditions used in the system. For example, Banerjee et al.[54] were able to control the binding of azo-ligand functionalized nanotube to cyclodextrines by UV light. In this context, dual-affinity avidin could serve as a connector or capture molecule between a biotinylated surface and biotinylated protein allowing reversible immobilization of proteins on the surface as demonstrated previously by Zhen et al.[55]

Avidin has been shown to induce chirality in certain chemical compounds linked to biotin,[56] as well as to catalyze the cleavage of certain chemical compounds.[48,57] The feasibility of modifying selected binding sites in dcAvd offers possibilities of generating avidins with high affinity to a solid support and simultaneously improved or novel catalytic properties in the modified domains. Furthermore, dcAvd could be used as a catalytic mold in chemical synthesis where the aim is to put together two reactive molecules at a low concentration. The potential of proteins in the design and development of novel catalytic tools is obvious.[58]

CONCLUSION

We demonstrated in this study that the dcAvd scaffold can be used to generate new avidins which show dual affinity toward biotin, the natural ligand of avidin. These proteins provide novel biotechnical tools that should find a role in a number of sophisticated techniques, including targeted drug delivery and nanotechnology, as well as in more conventional separation and purification or labelling protocols. Furthermore, these examples open the way to the introduction of more radical changes into the domains of dcAvd, such as changes in the ligand specificity, leading to novel applications.

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REFERENCES

1. Green NM. Avidin. Adv Protein Chem 1975;29:85–133.
2. Hendrickson WA, Pähler A, Smith JL, Satow Y, Merritt EA, Phizackerley RP. Crystal structure of core streptavidin determined from multiwavelength anomalous diffraction of synchrotron radiation. Proc Natl Acad Sci USA 1989;86:2190–2194.
3. Livnah O, Bayer EA, Wilchek M, Sussman JL. Three-dimensional structures of avidin and the avidin–biotin complex. Proc Natl Acad Sci USA 1993;90:5076–5080.
4. Pugliese L, Coda A, Malcovati M, Bolognesi M. Three-dimensional structure of the tetragonal crystal form of egg-white avidin in its functional complex with biotin at 2.7 Å resolution. J Mol Biol 1993;231:698–710.
5. Weber PC, Ohlendorf DH, Wendoloski JJ, Salemme FR. Structural origins of high-affinity biotin binding to streptavidin. Science 1989;243:85–88.
6. Wilchek M, Bayer EA. Foreword and introduction to the book (strept)avidin–biotin system. Biomol Eng 1999;16:1–4.
7. Hinds BJ, Chopra N, Rantell T, Andrews R, Gavalas V, Bachas LG. Aligned multiwalled carbon nanotube membranes. Science 2004;303:62–65.
8. Keren K, Berman RS, Buchstab E, Sivan U, Braun E. DNA-templated carbon nanotube field-effect transistor. Science 2003; 302:1380–1382.
9. Banerjee I, Yu L, Matsui H. Location-specific biological functionalization on nanotubes: attachment of proteins at the ends of nanotubes using Au nanocrustal masks. Nano Lett 2003;3:283–287.
10. Paganelli G, Grana C, Chinol M, Cremonesi M, De Cicco C, De Braud F, Robertson C, Zurrida S, Casadio C, Zoboli S, Siccardi AG, Veronesi U. Antibody-guided three-step therapy for high grade glioma with yttrium-90 biotin. Eur J Nucl Med 1999;26:348–357.
11. Lehtolainen P, Wirth T, Taskinen AK, Lehenkari P, Leppänen O, Lappalainen M, Pulkkanen K, Marttila A, Marjomäki V, Airenne KJ, Horton M, Kulomaa MS, Ylä-Herttuala S. Targeting of biotinylated compounds to its target tissue using a low-density lipoprotein receptor–avidin fusion protein. Gene Ther 2003;10: 2090–2097.
12. Parrott MB, Adams KE, Mercier GT, Mok H, Campos SK, Barry MA. Metabolically biotinylated adenovirus for cell targeting, ligand screening, and vector purification. Mol Ther 2003;8:688–700.
13. Räty JK, Airenne KJ, Marttila AT, Marjomäki V, Hytönen VP, Lehtolainen P, Laitinen OH, Mähönen AJ, Kulomaa MS, Ylä-Herttuala S. Enhanced gene delivery by avidin-displaying baculovirus. Mol Ther 2004;9:282–291.
14. Barry MA, Campos SK, Ghosh D, Adams KE, Mok H, Mercier GT, Parrott MB. Biotinylated gene therapy vectors. Expert Opin Biol Ther 2003;3:925–940.
15. Chilkoti A, Tan PH, Stayton PS. Site-directed mutagenesis studies of the high-affinity streptavidin-biotin complex: contributions of tryptophan residues 79, 108, and 120. Proc Natl Acad Sci USA 1995;92:1754–1758.
16. Sano T, Cantor CR. Intersubunit contacts made by tryphtophan 120 with biotin are essential for both strong biotin binding and biotin-induced tighter subunit association of streptavidin. Proc Natl Acad Sci USA 1995;92:3180–3184.
17. Reznik GO, Vajda S, Smith CL, Cantor CR, Sano T. Streptavidins with intersubunit crosslinks have enhanced stability. Nat Biotechnol 1996;14:1007–1011.
18. Sano T, Vajda S, Smith CL, Cantor CR. Engineering subunit association of multisubunit proteins: a dimeric streptavidin. Proc Natl Acad Sci USA 1997;94:6153–6158.
19. Reznik GO, Vajda S, Sano T, Cantor CR. A streptavidin mutant with altered ligand-binding specificity. Proc Natl Acad Sci USA 1998;95:13525–13530.
20. Chu V, Freitag S, Le Trong I, Stenkamp RE, Stayton PS. Thermodynamic and structural consequences of flexible loop deletion by circular permutation in the streptavidin–biotin system. Protein Sci 1998;7:848–859.
21. Marttila A, Airenne K, Laitinen O, Kulik T, Bayer E, Wilchek M, Kulomaa M. Engineering of chicken avidin: a progressive series of reduced charge mutants. FEBS Lett 1998;441:313–317.
22. Laitinen OH, Airenne KJ, Marttila AT, Kulik T, Porkka E, Bayer EA, Wilchek M, Kulomaa MS. Mutation of a critical tryptophan to lysine in avidin or streptavidin may explain why sea urchin fibropellin adopts an avidin-like domain. FEBS Lett 1999;461:52–58.
23. Freitag S, Le Trong I, Klumb LA, Chu V, Chilkoti A, Stayton PS, Stenkamp RE. X-ray crystallographic studies of streptavidin mutants binding to biotin. Biomol Eng 1999;16:13–19.
24. Marttila AT, Laitinen OH, Airenne KJ, Kulik T, Bayer EA, Wilchek M, Kulomaa MS. Recombinant NeutraLite avidin: a non-glycosylated, acidic mutant of chicken avidin that exhibits high affinity for biotin and low non-specific binding properties. FEBS Lett 2000;467:31–36.
25. Laitinen OH, Marttila AT, Airenne KJ, Kulik T, Livnah O, Bayer EA, Wilchek M, Kulomaa MS. Biotin induces tetramerization of a recombinant monomeric avidin: a model for protein–protein interactions. J Biol Chem 2001;276:8219–8224.
26. Marttila AT, Hytönen VP, Laitinen OH, Bayer EA, Wilchek M, Kulomaa MS. Mutation of the important Tyr-33 residue of chicken avidin: functional and structural consequences. Biochem J 2003; 369:249–254.
27. Nordlund HR, Laitinen OH, Uotila ST, Nyholm T, Hytönen VP, Slotte JP, Kulomaa MS. Enhancing the thermal stability of avidin: introduction of disulfide bridges between subunit interfaces. J Biol Chem 2003;278:2479–2483.
28. Nordlund HR, Hytönen VP, Laitinen OH, Uotila ST, Niskanen EA, Savolainen J, Porkka E, Kulomaa MS. Introduction of histidine residues into avidin subunit interfaces allows pH-dependent regulation of quaternary structure and biotin binding. FEBS Lett 2003;555:449–454.
29. Stayton PS, Shimoboji T, Long C, Chilkoti A, Chen G, Harris JM, Hoffman AS. Control of protein–ligand recognition using a stimuli-responsive polymer. Nature 1995;378:472–474.
30. Lackey CA, Murthy N, Press OW, Tirrell DA, Hoffman AS, Stayton PS. Hemolytic activity of pH-responsive polymer–streptavidin bioconjugates. Bioconjug Chem 1999;10:401–405.
31. Lackey CA, Press OW, Hoffman AS, Stayton PS. A biomimetic pH-responsive polymer directs endosomal release and intracellular delivery of an endocytosed antibody complex. Bioconjug Chem 2002;13:996–1001.
32. Chilkoti A, Schwartz BL, Smith RD, Long CJ, Stayton PS. Engineerd chimeric streptavidin tetramers as novel tools for bioseparations and drug delivery. Nat Biotechnol (previously Bio/Technol) 1995;13:1198–1204.
33. Nordlund HR, Laitinen OH, Hytönen VP, Uotila ST, Porkka E, Kulomaa MS. Construction of a dual-chain pseudotetrameric chicken avidin by combining two circularly permuted avidins. J Biol Chem 2004;279:36715–36719.
34. Klumb LA, Chu V, Stayton PS. Energetic roles of hydrogen bonds at the ureido oxygen binding pocket in the streptavidin–biotin complex. Biochemistry 1998;37:7657–7663.
35. Sarkar G, Sommer SS. The "megaprimer" method of site-directed mutagenesis. Biotechniques 1990;8:404–407.
36. Laitinen OH, Hytönen VP, Ahlroth MK, Pentikäinen OT, Gallagher C, Nordlund HR, Ovod V, Marttila AT, Porkka E, Heino S, Johnson MS, Airenne KJ, Kulomaa MS. Chicken avidin-related proteins show altered biotin-binding and physico-chemical properties as compared with avidin. Biochem J 2002;363:609–617.
37. Hytönen VP, Nyholm TK, Pentikäinen OT, Vaarno J, Porkka EJ, Nordlund HR, Johnson MS, Slotte JP, Laitinen OH, Kulomaa MS. Chicken avidin-related protein 4/5 shows superior thermal stability when compared with avidin while retaining high affinity to biotin. J Biol Chem 2004;279:9337–9343.
38. Gonzalez M, Argarana CE, Fidelio GD. Extremely high thermal stability of streptavidin and avidin upon biotin binding. Biomol Eng 1999;16:67–72.
39. Brandts JF, Lin LN. Study of strong to ultratight protein interactions using differential scanning calorimetry. Biochemistry 1990; 29:6927–6940.
40. Hamblett KJ, Kegley BB, Hamlin DK, Chyan MK, Hyre DE, Press OW, Wilbur DS, Stayton PS. A streptavidin–biotin binding system that minimizes blocking by endogenous biotin. Bioconjug Chem 2002;13:588–598.
41. Hytönen VP, Laitinen OH, Airenne TT, Kidron H, Meltola NJ, Porkka E, Hörhä J, Paldanius T, Määttä JA, Nordlund HR, Johnson MS, Salminen TA, Airenne KJ, Ylä-Herttuala S, Kulomaa MS. Efficient production of active chicken avidin using a

bacterial signal peptide in *Escherichia coli*. Biochem J 2004;384: 385–390.
42. Hytönen VP, Laitinen OH, Grapputo A, Kettunen A, Savolainen J, Kalkkinen N, Marttila AT, Nordlund HR, Nyholm TK, Paganelli G, Kulomaa MS. Characterization of poultry egg-white avidins and their potential as a tool in pretargeting cancer treatment. Biochem J 2003;372:219–225.
43. Bayer EA, Ehrlich-Rogozinski S, Wilchek M. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic method for assessing the quaternary state and comparative thermostability of avidin and streptavidin. Electrophoresis 1996;17:1319–1324.
44. Hyre DE, Le Trong I, Freitag S, Stenkamp RE, Stayton PS. Ser45 plays an important role in managing both the equilibrium and transition state energetics of the streptavidin–biotin system. Protein Sci 2000;9:878–885.
45. Green NM, Toms EJ. The properties of subunits of avidin coupled to sepharose. Biochem J 1973;133:687–700.
46. Piran U, Riordan WJ. Dissociation rate constant of the biotin–streptavidin complex. J Immunol Methods 1990;133:141–143.
47. Pazy Y, Kulik T, Bayer EA, Wilchek M, Livnah O. Ligand exchange between proteins: exchange of biotin and biotin derivatives between avidin and streptavidin. J Biol Chem 2002;277: 30892–30900.
48. Huberman T, Eisenberg-Domovich Y, Gitlin G, Kulik T, Bayer EA, Wilchek M, Livnah O. Chicken avidin exhibits pseudo-catalytic properties: biochemical, structural, and electrostatic consequences. J Biol Chem 2001;276:32031–32039.
49. Fersht AR, Shi JP, Knill-Jones J, Lowe DM, Wilkinson AJ, Blow DM, Brick P, Carter P, Waye MM, Winter G. Hydrogen bonding and biological specificity analysed by protein engineering. Nature 1985;314:235–238.
50. Weber PC, Wendoloski JJ, Pantoliano MW, Salemme FR. Crystallogaphic and thermodynamic comparison of natural and synthetic ligands bound to streptavidin. J Am Chem Soc 1992;114:3197–3200.
51. Laitinen OH, Nordlund HR, Hytönen VP, Uotila ST, Marttila AT, Savolainen J, Airenne KJ, Livnah O, Bayer EA, Wilchek M, Kulomaa MS. Rational design of an active avidin monomer. J Biol Chem 2003;278:4010–4014.
52. Bayer EA, Wilchek M. Application of avidin–biotin tecnology to affinity-based separations. J Chromatogr 1990;510:3–11.
53. Holmberg A, Blomstergren A, Nord O, Lukacs M, Lundeberg J, Uhlen M. The biotin–streptavidin interaction can be reversibly broken using water at elevated temperatures. Electrophoresis 2005;26:501–510.
54. Banerjee I, Yu L, Matsui H. Application of host–guest chemistry in nanotube-based device fabrication: photochemically controlled immobilization of azobenzene nanotubes on patterned alpha-CD monolayer/Au substrates via molecular recognition. J Am Chem Soc 2003;125:9542–9543.
55. Zhen G, Eggli V, Voros J, Zammaretti P, Textor M, Glockshuber R, Kuennemann E. Immobilization of the enzyme beta-lactamase on biotin-derivatized poly(L-lysine)-g-poly(ethylene glycol)-coated sensor chips: a study on oriented attachment and surface activity by enzyme kinetics and in situ optical sensing. Langmuir 2004;20: 10464–10473.
56. Collot J, Gradinaru J, Humbert N, Skander M, Zocchi A, Ward T. Artificial metalloenzymes for enantioselective catalysis based on biotin–avidin. J Am Chem Soc 2003;125:9030–9031.
57. Vetter S, Bayer EA, Wilchek M. Avidin can be forced to adopt catalytic activity. J Am Chem Soc 1994;116:9369–9370.
58. Xu Y, Yamamoto N, Janda KD. Catalytic antibodies: hapten design strategies and screening methods. Bioorg Med Chem 2004;12:5247–5268.