Tetravalent single-chain avidin: from subunits to protein domains via circularly permuted avidins

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scAvd (single-chain avidin, where two dcAvd are joined in a single polypeptide chain), having four biotin-binding domains, was constructed by fusion of topologically modified avidin units. scAvd showed similar biotin binding and thermal stability properties as chicken avidin. The DNA construct encoding scAvd contains four circularly permuted avidin domains, plus short linkers connecting the four domains into a single polypeptide chain. In contrast with wild-type avidin, which contains four identical avidin monomers, scAvd enables each one of the four avidin domains to be independently modified by protein engineering. Therefore the scAvd scaffold can be used to construct spatially and stoichiometrically defined pseudotetrameric avidin molecules

showing different domain characteristics. In addition, unmodified scAvd could be used as a fusion partner, since it provides a unique non-oligomeric structure, which is fully functional with four high-affinity biotin-binding sites. Furthermore, the subunit-to-domain strategy described in the present study could be applied to other proteins and protein complexes, facilitating the development of sophisticated protein tools for applications in nanotechnology and life sciences.

Key words: avidin–biotin technology, circular permutation, dual-chain avidin, protein engineering, single-chain avidin, subunit fusion.

INTRODUCTION

Chicken avidin displays extreme affinity towards biotin and has therefore become a widely used protein tool in life sciences [1]. Together with its bacterial analogue, streptavidin, avidin has found uses in techniques ranging from traditional labelling, separation and purification methods [2] to highly specialized fields in targeted medicine, molecular self-assembly and nanotechnology [3,4].

Recombinant forms of avidin and streptavidin, or shortly (strept)avidin, can be efficiently produced both by eukaryotic and prokaryotic expression systems [5–9]. This has facilitated mutagenesis studies of these proteins, providing fundamental knowledge of their unique structure–function relationships [10,11], and also produced novel tools for avidin–biotin technology. For example, the properties of charge [12,13], oligomerization [14–16], glycosylation [17], thermal stability [18–20], biotin binding [10,21–24], biodistribution [25] and ligand specificity [26] have been modified. These modifications have markedly diversified the protein and ligand repertoire of the (strept)avidin–biotin system [1,27], which is also reflected by the number of commercially available avidin–biotin products.

Avidin and streptavidin are homotetrameric proteins encoded by single genes [28,29]. This property stringently restricts their genetic modification, since each of the genetically applied modifications will uniformly be present in all four subunits of the re-

sulting mutant protein tetramer. Two attempts to overcome this limitation have been presented, the earlier of which was based on mixing two different types of denatured streptavidins, followed by renaturation leading to the formation of various combinations of homo- and hetero-tetramers [30]. This system was further improved by engineering the subunit interfaces leading to more controlled, but not completely defined, stoichiometric distribution of the different subunits [19]. In a more recent approach, we radically engineered the topology of avidin [31]. In that study, two different circularly permuted avidin variants were generated first and were subsequently joined in one polypeptide chain. This novel dcAvd (dual-chain avidin, where the circularly permuted avidins cpAvd5 → 4 and cpAvd6 → 5 are joined in a single polypeptide chain) scaffold spontaneously formed dimers (pseudotetramers, i.e. four binding sites per dimeric quaternary structure unit) in solution, showing functional and structural characteristics equivalent to those of wt (wild-type) avidin [31]. Although dcAvd proved to be a powerful engineering scaffold, its innate structural symmetry serves as a source of heterogeneity at the quaternary structure level. The components of dcAvd may form a dimeric complex in two different ways and only the large one-to-four interface [32] is unquestionably fixed. The exact position of the applied modification in the quaternary structure cannot thus be perfectly adjusted. In order to overcome this, we have recently planted asymmetrical but structurally complementary patterns

Abbreviations used: Avd, chicken avidin protein; cpAvd5 → 4 domain, circularly permuted avidin domain, where the new N-terminus is before β -strand 5 and the new C-terminus after β -strand 4; cpAvd6 → 5 domain, circularly permuted avidin domain, where the new N-terminus is before β -strand 6 and the new C-terminus after β -strand 5; dcAvd, dual chain avidin, where the circularly permuted avidins cpAvd5 → 4 and cpAvd6 → 5 are joined in a single polypeptide chain; DSC, differential scanning calorimetry; scAvd, single-chain avidin, where two dcAvds are joined in a single polypeptide chain; scFv, single-chain Fv; wt, wild-type.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession number AJ966780.

Table 1 Structural and biochemical properties of avidins

Heat-induced unfolding (T_m) was analysed by DSC. Apparent molecular masses were determined by gel filtration. Biotin-binding stoichiometry was determined by quenching of intrinsic fluorescence. Dissociation of fluorescent biotin was analysed at two different temperatures.

	DSC		Gel filtration		Free biotin-binding sites		Dissociation of fluorescent biotin			
	T_m (°C)		Elution time (min)	Molecular mass (kDa)	Number of sites	Fluorescence quenching (350 nm) (%)	k_{diss} (s ⁻¹) at 23 °C	Release at 1 h (%)	k_{diss} (s ⁻¹) at 50 °C	Release at 1 h (%)
	– Biotin	+ Biotin								
Avidin	83.5 ± 0.1*	117.0 ± 0.7*	31.5	58.4	3.34	47.7	2.0 × 10 ⁻⁵	10.7	2.4 × 10 ⁻⁴	71.5
dcAvid	80.2 ± 0.0†	115.9†	32.9	40.7	3.86	51.4	1.5 × 10 ⁻⁵	10.1	2.7 × 10 ⁻⁴	67.4
scAvid	83.1 ± 0.1	114.0 ± 1.2	31.8	54.0	3.20	44.4	1.3 × 10 ⁻⁵	8.8	2.5 × 10 ⁻⁴	62.3

* From [34].

† From [39].

into dcAvid, which should facilitate formation of the desired fixed conformation (V. P. Hytönen, J. Hörhä, K. Helttunen, E. Niskanen, M. S. Kulomaa and H. R. Nordlund, unpublished work). In this conformation, the termini of the two dcAvid monomers reside on opposite faces of the pseudotetramer. However, by utilizing the dcAvid scaffold, it is not possible to obtain four different domains or to modify, for example, only one of the four binding sites or domain surfaces per quaternary structure unit.

In the present study, we continued the topology engineering of avidin, by constructing an avidin scaffold in which all four subunits are joined in a single polypeptide chain, as domains. The scAvid (single-chain avidin, where two dcAvid are joined in a single polypeptide chain) molecule produced displayed functional and structural characteristics similar to those of wt avidin. Because a single polypeptide chain forms all four binding sites in this protein, it provides novel potential for controlled modifications anywhere in the protein.

EXPERIMENTAL

Materials

Biotin was obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). Chicken avidin, a gift from Belovo (Bastogne, Belgium), was used as a control protein in the analyses. The concentrations of avidin solutions were calculated from the absorbance A at 280 nm using a molar absorption coefficient (ϵ) of 24 280 M⁻¹ · cm⁻¹ per binding site.

Design of the expression construct of scAvid and production of the recombinant proteins

A construct encoding dcAvid in pFASTBAC1 was modified using the QuikChange method (Stratagene, La Jolla, CA, U.S.A.) so that the BamHI restriction site in the peptide linker connecting the original circularly permuted avidin cDNAs was destroyed without changing the polypeptide sequence. The coding region obtained was extended by PCR to remove the stop codon and to add part of the 12 amino acid residue linker (GGSGSGSGSGSG) and also a BamHI cloning site at the 3'-end. In another PCR, the QuikChange-modified dcAvid core region (without the DNA encoding the signal peptide) was extended to add the rest of the 12 amino acid linker and a BamHI site at the 5'-end. The PCR products were then successively cloned, tail-to-head, into pFASTBAC1 vector and confirmed by sequencing. The sequences of scAvid (accession number AJ966780) and dcAvid (accession number AJ616762; [31]) have been deposited in the EMBL database.

Recombinant baculoviruses encoding scAvid were generated using the pFASTBAC1-derivative vector described above accord-

ing to the Bac-To-Bac™ manufacturer's instructions (Gibco BRL, Life Technologies, Gaithersburg, MD, U.S.A.). Proteins were produced in baculovirus-infected Sf9 insect cells in a biotin-free medium as reported previously [33]. The scAvid protein was purified in a single step by affinity chromatography on a 2-imino-biotin–agarose column, as described previously [15].

Radiobiotin dissociation assay

The dissociation rate constant of [³H]biotin from scAvid was determined by competition with free biotin at various temperatures as described in detail elsewhere [21]. Sodium phosphate buffer (50 mM; pH 7.0) containing 100 mM NaCl was used in the measurements. A global fit approach was used to analyse the data by the method of Hyre et al. [24].

Dissociation analysis using fluorescent biotin

Dissociation of a fluorescent biotin conjugate from scAvid by competition with free biotin was measured using a PerkinElmer LS55 luminometer. The measurements were performed in sodium phosphate buffer (50 mM; pH 7.0) containing 650 mM NaCl at 25 and 50 °C as described previously [9]. Avidin and dcAvid were also analysed as reference proteins.

Data were analysed using a one-phase dissociation model as described previously [9]. The dissociation rate constant (k_{diss}) was determined by fitting the equation $-k_{\text{diss}}t = \ln(B/B_0)$ to the data, in which B_0 is the maximum binding measured (100 %) and B the amount of complex measured as a function of time. In the case of dissociation rate constant measurement, the first 500 s were omitted from the data to abolish the effect of the fast initial phase characteristic of the avidin–BF560–biotin interaction.

Determination of the number of free biotin-binding sites

The number of free binding sites per scAvid molecule was determined by monitoring the quenching of intrinsic protein fluorescence upon biotin binding. This value was also measured for dcAvid and wt avidin. The protein samples were diluted to 500 ± 15 nM in a final volume of 2 ml in sodium phosphate buffer (50 mM; pH 7.0) containing 650 mM NaCl, and titrated with 1 µl addition of 70 µM biotin in the same buffer. The protein sample was continuously mixed by a magnetic stirrer in the cuvette.

Emission intensity at 350 nm (5 nm slit) was measured by exciting the sample at 280 nm (2.5 nm slit). The fluorescence intensity from each successive biotin concentration was averaged over an 8 s equilibrium signal measurement. The end point of the quenching was determined by fitting a line to the data, and by determining the total quenching by averaging the last three measured intensities (Figure 4 and Table 1).

DSC (differential scanning calorimetry)

The thermostability of scAvd was studied using DSC as described previously [34]. The T_m of protein unfolding was determined both in the absence and presence of biotin (3:1, biotin-binding site) with a protein concentration of 0.21–0.23 mg/ml in sodium phosphate buffer (50 mM; pH 7.0) containing 100 mM NaCl. The thermograms (25–130 °C, 0.92 °C/min) were collected with a Nano II high-sensitivity scanning calorimeter (Calorimetry Sciences, Lindon, UT, U.S.A.) and analysed using CpCalc 2.1 (Calorimetry Sciences) and Microcal Origin 6.0 software (OriginLab, Northampton, MA, U.S.A.).

Microplate stability assay

The thermal stability of scAvd was studied by a microplate assay. Proteins were diluted to a final concentration of 5 µg/ml in sodium phosphate (50 mM; pH 7.0) containing 100 mM NaCl, and transferred into PCR tubes. Samples were incubated at 80, 90 or 100 °C for 2–32 min. After the heat treatment, the tubes were kept on ice. The protein samples were transferred to a 96-well Nunc Maxisorp plate and incubated at 37 °C for 1.5 h. After incubation, the wells were washed three times with PBS–Tween (Tween 20; 0.05 %, v/v) and blocked with BSA (1 %, w/v) in PBS for 1 h at 37 °C. The wells were washed again three times with PBS–Tween before incubation with biotinylated alkaline phosphatase (Pierce) at 37 °C for 1 h. Once again, the wells were washed with PBS–Tween and *p*-nitrophenyl phosphate in diethanolamine buffer (1 mg/ml) was applied to the wells. A_{405} was measured using a plate reader.

Gel-filtration analysis

The size of scAvd in solution was measured by FPLC gel filtration at room temperature (21–23 °C), as described previously [35]. Sodium phosphate buffer (50 mM; pH 7.0) containing 650 mM NaCl was used as the liquid phase.

Molecular modelling of scAvd

A molecular model of scAvd was generated using MODELLER, release 6v6 [36]. A manual alignment of circularly permuted avidin amino acid sequences and the linker regions was modelled on to the avidin template structure, PDB code 2AVI [32]. The model was visually inspected for stereochemical errors and integrity of the biotin-binding sites. Figures were prepared using PyMOL [37].

RESULTS

Protein production and purification

scAvd produced in insect cells was efficiently purified from cell extracts by 2-iminobiotin affinity chromatography, indicating that it was soluble and biologically active with respect to ligand binding.

In FPLC gel-filtration analysis, the purified scAvd eluted essentially at the same volume as wt avidin, indicating similar molecular mass in solution (Table 1). This proved that scAvd had folded and formed the quaternary structure assembly as designed (Figure 1), and that it existed as a homogenous pseudotetrameric protein (i.e. four binding sites/polypeptide chain) without any mixed chain oligomeric forms. Purified scAvd migrated in SDS/PAGE analysis as a 65 kDa major band (Figure 2), which confirmed the pseudotetrameric assembly, as only trace quantities of smaller proteins were observed on the gel. The two forms

slightly different in mass of the protein were most probably caused by differential glycosylation or the extent of sugar moiety processing in insect cells, as has also been reported previously for wt recombinant avidin [6]. Only traces of approx. 30 kDa forms were detected, indicating high homogeneity of the purified protein. Western-blot analysis with polyclonal anti-avidin (University of Oulu, Finland) revealed identical molecular mass bands with those shown by Coomassie Blue staining (Figure 2), but, being a more sensitive assay, highlighted the small amount of possibly cleaved scAvd (protease inhibitors were not included in the purification method).

Radiobiotin dissociation assay

The radiobiotin assay was performed at various temperatures (Figure 3). Like avidin and dcAvd, scAvd exhibited extremely slow dissociation of [³H]biotin. The dissociation rate constants for scAvd were somewhat lower compared with those of Avd (chicken avidin protein) and dcAvd.

Fluorescent biotin conjugate dissociation assay

The dissociation assay with ArcDiaTM BF560 fluorescent biotin [9] showed similar properties for all the avidin proteins. Both dcAvd and scAvd showed slightly lower dissociation rates and lower total release values at 25 °C when compared with wt avidin. The results obtained were in general agreement with those described above for the radiobiotin assay. Dissociation rate constants and total release values after 1 h competition with excess free biotin are shown in Table 1.

Determination of the number of biotin-binding sites

Quenching of avidin intrinsic fluorescence upon biotin binding was used to determine the number of free binding sites [38]. scAvd showed almost the same binding capacity (3.2 binding sites/molecule) compared with wt avidin (3.3 binding sites/tetramer) (Table 1). A similar difference was also detected in the total quenching end point values, since 44.4 % of the emission intensity of scAvd at 350 nm was quenched by successive additions of biotin compared with 47.7 % for avidin (Figure 4 and Table 1). In agreement with these results, the quenching for the whole emission spectrum (290–500 nm) owing to biotin binding was 44.9 % for avidin, 39.7 % for scAvd and 45.1 % for dcAvd.

Avidin stability by DSC

scAvd showed high thermal stability in DSC analysis. The T_m values obtained are similar to those previously measured for avidin [34] and dcAvd [39]. Addition of biotin stabilized scAvd, as seen for the other avidin forms (Table 1).

Stability of avidins determined by microplate assay

Both scAvd and dcAvd showed rapid inactivation in the assay performed at 100 °C. Avidin control showed a significantly slower response to the heat treatment (results not shown). When the temperature was lowered to 90 °C, scAvd and dcAvd still exhibited a relatively fast loss of activity, dcAvd showing slightly higher stability at 4 and 8 min time points when compared with scAvd. Wt avidin showed slow inactivation at this temperature and more than 50 % of the activity was present after 32 min of treatment (Figure 5). At 80 °C, all the avidins showed slow inactivation. The activity of scAvd after 32 min of treatment at 80 °C was 26 %, whereas those measured for avidin and dcAvd were 82 and 59 % respectively (Figure 5).

Figure 1 Design of scAvd

(A) Topology of scAvd. The secondary-structure elements of avidin are shown and numbered with respect to wt avidin, and the positions of artificial linkers are shown. **(B)** Molecular model of scAvd based on the crystal structure of avidin (PDB code 2AVI). The 12-residue linker between dcAvd units is shown in yellow. The linkers connecting circularly permuted avidins in dcAvd units are shown in green. Biotin ligands are shown in space-filled CPK coloured representation. The cpAvd5 → 4 domains are shown in red and the cpAvd6 → 5 domains in blue.

Figure 2 Purification of scAvd

Coomassie Blue-stained SDS/PAGE analysis of elution fractions 1–5 after 2-iodinobiotin purification. Lane M, molecular mass standards (Bio-Rad low mass). WB, Western-blot analysis with polyclonal anti-avidin.

DISCUSSION

Many important protein tools in life sciences are oligomeric, including for example antibodies, several enzymes and

Figure 3 Temperature dependence of biotin dissociation rates measured by the [³H]biotin dissociation assay

Radiobiotin dissociation rate constants values measured for the proteins are plotted as a function of temperature. The models for dissociation rate constants obtained by global fit analysis [24] are shown as lines and the measured individual dissociation rates as symbols. The dissociation rate constants values determined previously for avidin and dcAvd are also shown [39].

Figure 4 Quenching of intrinsic protein fluorescence by biotin binding

Protein sample was titrated with *D*-biotin. The sample was excited at 280 nm and emission intensity was measured at 350 nm. The number of free biotin-binding sites in the proteins was determined from the data.

Figure 5 Temperature sensitivity determined by the microplate assay

The protein sample was kept at the indicated temperature and samples were taken at designated time points and stored on ice. Binding of biotinylated alkaline phosphatase was then measured.

(strept)avidin. Oligomeric proteins are somewhat challenging for biotechnology, since the quaternary structure integrity must be maintained in an assay to preserve function. Functionally independent domains are commonly used building blocks in proteins. Some proteins with the same function and equivalent overall structure are found as subunits in one species and as domains in another [40]. In some cases, the domain forms are more suitable targets for protein engineering, providing an opportunity to fully control modifications at the quaternary structure level.

In order to form a molecule with four biotin-binding domains in the same polypeptide chain, in the present study we combined two dcAvid units by a tail-to-head fusion strategy using a 12 amino acid linker peptide. In scAvid, the pseudotetrameric molecule derives from its continuous DNA sequence, not from post-translational assembly of four identical peptides. Therefore its binding domains can be independently engineered using site-directed mutagenesis for example.

In principle, the design of scAvid was based on two central issues. First, we determined the length of the dcAvid–dcAvid linker to be short enough to allow only one type of quaternary structure formation, leading to single-chain (non-mixed) protein products. Secondly, the linker was adjusted to be long enough to allow ligand binding, in particular at the first cpAvid6 → 5 domain (circularly permuted avidin domain, where the new N-terminus is before β -strand 6 and the new C-terminus after β -strand 5), which was connected by the linker to the second cpAvid5 → 4 domain (circularly permuted avidin domain, where the new N-terminus is before β -strand 5 and the new C-terminus after β -strand 4) close to its binding site entrance (Figure 1). Inspection of the modelled scAvid structure indicated that this dcAvid–dcAvid connection site was clearly the most crucial and demanding detail of the engineered protein with respect to both structure and function. Previous studies with scFv (single-chain Fv) antibodies have provided insights into the interdomain linker lengths and types [41,42], which could also be utilized for further scAvid engineering. For example, a linker shorter than that used in the present study could produce a dimeric scAvid assembly comprising eight binding sites formed by two linker restricted scAvid molecules, in which certain intramolecular interface formations could be designed to be unfavoured or impossible.

Skerra [43] utilized lipocalins, termed as anticalins, successfully as scaffold proteins to develop novel binding affinities for several different ligands. Similarly, our dcAvid and especially scAvid scaffolds are suitable framework proteins to exhibit variable specificities for two or even up to four different ligands. At best, the affinities of the engineered lipocalins for their novel ligands are in the nanomolar range. Avidin exhibits femtomolar affinity for its natural ligand biotin. If a similar high affinity could be engineered for other small non-biotin ligands, very useful molecules could be created using dcAvid or scAvid to display these new ligand-binding domains together with biotin-binding domains. Moreover, scAvid is relatively small for a protein capable of tetravalency. It has a molecular mass close to the diabody scFvs, which are only bivalent (63 kDa versus 60 kDa) [44]. Such a small mass combined with higher valency could be a benefit in applications.

One potential application for scAvid could be fusion proteins. Genetic fusions with normal tetrameric avidin can be problematic if the desired fusion partner is already oligomeric or is a membrane protein. In fusion approaches, scAvid can be considered to be stoichiometrically analogous to the previously developed monoavidin [16]. Because the affinity of scAvid for biotin is several orders of magnitude stronger than that of monoavidin, it would be a valuable fusion tag in situations where extreme affinity is required.

A marked benefit of avidin as a scaffold protein, compared with other scaffold candidates, is its extraordinary stability under various demanding conditions. It is noteworthy that the thermal stability of scAvid, according to DSC analysis, was almost equal to that of wt avidin. In the presence of the ligand, however, a T_m value 3° lower was determined for scAvid compared with wt protein. This difference may have some practical significance in high temperature applications as seen in the microplate assay (Figure 5). It could be possible to improve the thermal stability of scAvid scaffold by introducing the same mutations that we previously used to enhance the stability of avidin [18,20] and dcAvid [39], rendering scAvid more suitable for very high temperature conditions.

In order to summarize the value of scAvid as a potent binding scaffold, we can highlight the following aspects. First, scAvid provides a unique tetravalent framework for future modifications, possibly leading to avidins showing different affinities for one

ligand in different binding sites, or even multiple specificities for up to four different ligands. The principle of independent modification of the binding sites in dcAvid (containing two biotin-binding sites in a polypeptide chain) has been proven in another of our studies, where affinity-decreasing mutations were targeted to a selected binding site only [39]. This approach could be used to modify scAvid and, moreover, it offers an effective tool to also apply other modifications to the selected avidin domains. This approach could also be used more generally with other oligomeric proteins. Secondly, scAvid is also markedly more stable than many other scaffold candidates. Finally, scAvid presents a new stoichiometric alternative for avidin fusion tag systems. It may acquire the role as a key component in next-generation applications in biomedical sciences and nanotechnology.

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