Åbo Akademi University

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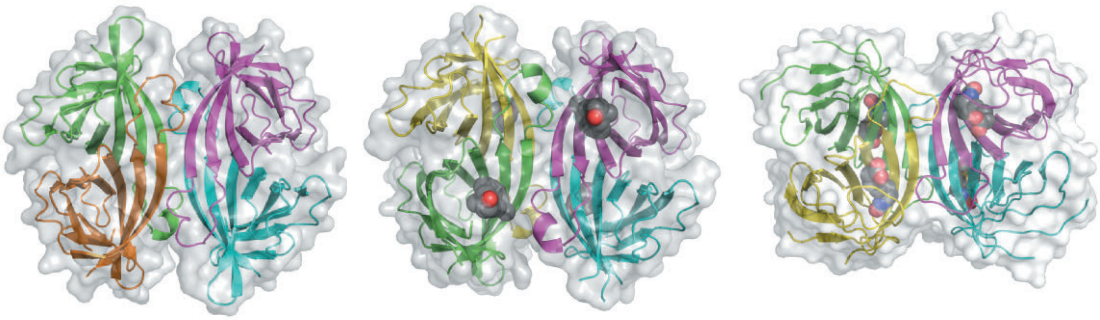


# Structural characterization of avidins with novel binding properties

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Biochemistry, Faculty of Science and Engineering
Åbo Akademi University
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From Biochemistry, Faculty of Science and Engineering, Åbo Akademi University, and National Doctoral Programme in Informational and Structural Biology

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**Cover:** From left: Surface/cartoon representation of the X-ray structures of apo-sbAvd-2(I117Y) [PDB:4U46], sbAvd-2(I117Y)—progesterone complex [PDB: 5LUR] and core-bradavidin—biotin complex [PDB:4BBO]. The figures were created using PyMol 1.3. Image by Nitin Agrawal.

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*Fascinating things await you,*
*if you dare to look*

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# Abstract

Avidin (Avd) is a protein first discovered in chicken egg white, and later in other birds, reptiles, amphibians and lancelet. Apart from these eukaryotic species, Avd is also identified in few fungal and in many bacterial species, the most well-known being streptavidin from *Streptomyces avidinii*. Avds are highly stable, homotetrameric – and in some cases dimeric – proteins, known for their extremely tight binding affinity to biotin; $K_d \sim 10^{-15}$ M for chicken Avd is the highest affinity observed for noncovalent ligand binding to a macromolecule. Due to the high affinity of Avds for biotin, the Avd-biotin binding system has been utilized in numerous life sciences applications in the field of biotechnology, medical diagnostics and nanotechnology.

Our collaborators have earlier developed circularly permuted, engineered Avd forms, dual-chain (dc) and single-chain (sc) Avds, which contain two and four, respectively, Avd subunits incorporated into one polypeptide chain. Each "domain" in dc and sc Avd thus corresponds to a monomer in wt Avd and have fully functional, high affinity biotin-binding site, which can be independently modified. The aim of this thesis was to structurally characterize novel Avd proteins, both natural and engineered forms, which bind non-biotin ligands, *e.g.* peptide ligands, steroids, and other small molecules. This provides the opportunity to create engineered dc or sc Avds, containing two or four distinct Avd domains having varied ligand-binding affinities for biotin, or non-biotin ligands. This thesis focuses on the structure determination of several Avds that recognize ligands different from biotin, which could be incorporated into engineered dc and sc Avds with a mixture of ligand preferences.

A number of artificial chicken Avd-based proteins, collectively called 'antidins', were designed through random and specific mutations and selected to bind ligands other than biotin. An engineered steroid-binding antidin, binding progesterone with micromolar affinity, was selected for crystallization trials. We solved the crystal structures of the steroid-binding antidin, sbAvd-2 (I117Y) without [Protein Data Bank code (PDB):4U46] and with [PDB:5LUR] progesterone bound to each monomer of the

tetramer. We also modelled several high-affinity progesterone-binding antidins possessing nanomolar affinity for the progesterone ligand, and performed computational docking experiments on them to assess their complexes with progesterone and biotin. This enabled us to evaluate possible interactions of antidins with these small molecules, even without their X-ray structures.

The natural tetrameric Avd, bradavidin from *Bradyrhizobium diazoefficiens*, a nitrogen-fixing bacterium present in the root nodules of soybean, provides novel opportunities for biotechnological uses of Avds because the wild-type (wt) bradavidin has a unique intrinsic C-terminal peptide ligand known as 'Brad-tag' bound to the binding site of the neighboring subunit. We have solved the X-ray structure of core-bradavidin – an engineered form of wt bradavidin, where the C-terminal residues Gly114-Lys138 have been truncated, including the Brad-tag residues Gly129-Lys138 – in complex with biotin [PDB:4BBO]. Comparison of the wt bradavidin—Brad-tag and core-bradavidin—biotin binding mode elucidated the conformational changes required in the bradavidin binding site to accommodate the two different ligands (Brad-tag *vs.* biotin). To better understand the mechanism and usefulness of Brad-tag as an intrinsic ligand, we also modelled the structure of two proteins containing the Brad-tag sequence and which likely binds it: rhodavidin from *Rhodopseudomonas palustris*, and bradavidin A2 from *Bradyrhizobium sp*. Ai1a-2.

The Avds detailed in this thesis include natural Avds binding intrinsic peptide ligands and artificial Avds, created through engineering or random mutagenesis, binding steroids or other small molecules. This study provides opportunities to design Avd-based scaffolds with tailor-made affinities for novel non-biotin ligands. These "domains" can be envisioned to be utilized as monomers *per se* in dimeric or tetrameric Avds, or as domains integrated into dc or sc Avds, designed for various bio(nano)technological applications in which simultaneous recognition and/or crosslinking of multiple molecular entities is advantageous.

# Abstract in Swedish

Avidin (Avd) är ett protein som först upptäcktes i kycklingäggvita och senare också i andra fåglar, reptiler, amfibier och lansettfiskar. Bortsett från dessa eukaryota arter finns Avd också i några svampar och i många bakteriearter. Det Avd som är bäst känt är streptavidin från *Streptomyces avidinii*. Avds är mycket stabila homotetramerer eller i vissa fall dimerer och är kända för sin extremt höga bindningsaffinitet för biotin, vilken för Avd från kyckling är $K_d \sim 10^{-15}$ M och därmed den högsta affiniteten som observerats för icke-kovalent ligandbindning till en makromolekyl. På grund av Avds höga affinitet för biotin har Avd-biotinbindningssystemet använts i många biovetenskapliga tillämpningar inom bioteknik, medicinsk diagnostik och nanoteknik.

Våra medarbetare har tidigare konstruerat cirkulärt permuterade Avd-former med dubbla kedjor (dc, *eng. dual-chain*) och enkla kedjor (sc, *eng. single-chain*), som innehåller två respektive fyra Avd underenheter införlivade i en polypeptidkedja. Varje "domän" i dc och sc Avd motsvarar sålunda en monomer i vildtyps (wt, *eng. wild type*) Avd och har ett fullt fungerande biotinbindande ställe med hög affinitet som kan modifieras oberoende av den övriga molekylen. Syftet med denna avhandling var att strukturellt karakterisera nya naturliga och manipulerade former av Avd proteiner som binder andra ligander än biotin, t.ex. peptidligander, steroider och andra småmolekyler. Detta ger möjlighet att skapa manipulerade dc eller sc Avds som innehåller två eller fyra distinkta Avd domäner med varierande bindningsaffinitet för biotin eller andra ligander. Denna avhandling fokuserar på strukturbestämning av flera Avds som känner igen ligander som skiljer sig från biotin och som kan införlivas i konstruerade dc och sc Avds för att uppnå en blandning av ligandspecificiteter.

Ett antal artificiella proteiner baserade på Avd från kyckling utformades genom slumpmässiga och specifika mutationer och de utvalda proteinerna binder andra ligander än biotin. Dessa proteiner kallas kollektivt "antidiner". Ett konstruerat steroidbindande antidin som binder progesteron med mikromolär affinitet valdes för kristalliseringsförsök. Vi

löste kristallstrukturerna för det steroidbindande antidinet sbAvd-2 (I117Y) utan [Protein Data Bank Code (PDB): 4U46] och med [PDB: 5LUR] progesteron bundet till varje monomer i tetrameren. Vi modellerade också strukturen hos flera progesteronbindande antidiner som har hög nanomolär affinitet för progesteron och utförde datorbaserade dockningsexperiment för att studera deras samverkan med progesteron och biotin. Därmed kunde vi utvärdera möjliga samverkningar mellan antidiner och dessa småmolekyler även utan tillgängliga strukturer.

*Bradyrhizobium diazoefficiens* är en kvävefixeringsbakterie i sojabönornas rotknutar och dess naturliga tetrameriska bradaividin ger nya möjligheter till biotekniska användningar av Avds, eftersom det har en unik inre C-terminal peptidligand känd som "Brad-tag" och som binder till bindningsstället hos den närliggande underenheten. Vi har löst röntgenstrukturen hos komplexet mellan biotin och kärnbradavidin [PDB: 4BBO] som är en konstruerad form av wt bradavidin där de C-terminala aminosyrorna Gly114-Lys138 har tagits bort. Denna del omfattar också aminosyrorna Gly129-Lys138 som utgör Brad-tag. Jämförelse av hur Brad-tag är bunden till wt bradavidin och biotin till kärnbradavidin belyser de konformationsändringar som krävs i bindningsstället hos bradavidin för att de två olika liganderna ska kunna binda. För att bättre förstå mekanismen och användbarheten av denna inre Brad-tag modellerade vi också strukturen hos två proteiner som innehåller aminosyrasekvensen för Brad-tag och som därmed sannolikt binder den. Dessa proteiner är rhodavidin från *Rhodopseudomonas palustris* och bradavidin A2 från *Bradyrhizobium sp.* Ai1a-2.

De Avds som beskrivs i denna avhandling omfattar naturliga Avds som binder inre peptider som ligander och artificiellt konstruerade Avds eller Avds som skapats genom slumpmässig mutagenes och som binder steroider eller andra småmolekyler. Denna studie gör det möjligt att utforma Avd-baserade proteiner med en stomme som har skräddarsydd affinitet för nya ligander som inte är biotin. Dessa "domäner" kan antas bilda dimerer eller tetramerer eller även fungera som domäner integrerade i dc eller sc Avds som har utformats för olika biologiska eller nanotekniska användningsområden där samtidig igenkänning och/eller tvärbindning av flera molekylenheter är fördelaktigt.

# List of Original Publications

I. Lehtonen SI, Tullila A*, **Agrawal N***, Kukkurainen S, Kähkönen N, Koskinen M, Nevanen, TK, Johnson MS, Airenne TT, Kulomaa MS, Riihimäki TA, Hytönen VP. (2016) Artificial avidin-based receptors for a panel of small molecules. *ACS Chemical Biology 11(1):211-21.*

II. **Agrawal N***, Lehtonen SI*, Uusi-Mäkelä M, Jain P, Viitala S, Määttä JAE, Kähkönen N, Riihimäki TA, Kulomaa MS, Johnson MS, Hytönen VP, Airenne TT. (2018) Molecular features of steroid-binding antidins and their use for assaying serum progesterone. *ChemBioChem, submitted manuscript.*

III. **Agrawal N**, Määttä JAE, Kulomaa, MS, Hytönen VP, Johnson M.S, Airenne TT. (2017) Structural characterization of core-bradavidin in complex with biotin. *PLoS One 12(4):e0176086.*

*Equal contribution

## Contributions of the Author

I. The author was responsible for determining the crystal structure of the sbAvd-2 (I117Y) [PDB: 4U46] antidin. The author was also responsible for writing part of the article.

This article has been published earlier as a part of the PhD thesis of Soili I. Lehtonen at the University of Tampere.

II. The author was responsible for determining the crystal structure of the sbAvd-2 (I117Y) – progesterone complex [PDB: 5LUR] antidin, and for performing the computational docking analysis of sbAvd-7–9 with progesterone and biotin. The author was also responsible for conceptualization, visualization and writing of the article with Soili I. Lehtonen and the other listed authors.

III. The author was responsible for characterizing the crystal structure of the core-bradavidin-biotin complex [PDB:4BBO], designing core-bradavidin mutants and for producing and analyzing computational models of rhodavidin and bradavidin A2. The author was also responsible for conceptualization, data curation, analysis, investigation, methodology, validation, visualization and writing of the article with other listed authors.

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Nitin Agrawal
Turku, August 2018

# Abbreviations

| | |
|---|---|
| 3D | Three-dimensional |
| Avd | Avidin |
| Avd-Btn | Avidin-biotin |
| AVR | Avidin-related protein |
| BBP | Biotin-binding protein |
| BSA | Bovine serum albumin |
| BSO | D-biotin D-sulfoxide |
| Btn | Biotin |
| cpAvd | Circularly permuted avidin |
| dcAvd | Dual-chain avidin |
| HABA | 4-hydroxyazobenzene-2-carboxylic acid |
| hbAvd | Hydrocortisone-binding avidin |
| HSA | Human serum albumin |
| H-bond | Hydrogen bond |
| mpAvd | Multipurpose avidin |
| PBS | Phosphate buffered saline |
| PCA | Principal component analysis |
| PDB | Protein Data Bank |
| PEG | polyethylene glycol |
| RMSD | Root-mean-squared deviation |
| sbAvd | Steroid-binding avidin |
| scAvd | Single-chain avidin |
| (Strept)avidin | Chicken avidin and streptavidin |
| wt | Wild-type |

# 1. Introduction

Avidins (Avds) belong to the calycin superfamily. Other members of the calycin protein superfamily include *e.g.* lipocalins, fatty acid binding proteins, metalloprotease inhibitors and triabin (Flower et al., 2000). Chicken egg-white avidin (Avd) (Eakin et al., 1941; György and Rose, 1941), and the homologous protein streptavidin from the bacterium *Streptomyces avidinii* (Chaiet and Wolf, 1964; Tausig and Wolf, 1964) are homotetrameric proteins that bind up to four molecules of vitamin B7 or vitamin H but commonly known as biotin (Btn). Both chicken Avd and streptavidin bind Btn with a high affinity, ($K_d$~$10^{-15}$ M for chicken Avd) (Green, 1975). This exceptional affinity for Btn has made the Avd-Btn complex a key tool in various life science applications *e.g.* immunological assays, hybridization assays, affinity chromatography, and has thus given rise to chicken Avd-Btn and streptavidin-Btn based technologies (Barnard et al., 1986; Bayer and Wilchek, 1990; Diamandis and Christopoulos, 1991; Wilchek and Bayer, 1990), referred jointly as strept(avidin)-Btn technology.

The strept(avidin)-Btn technology was further enhanced by the discovery of other natural avidins (Avds): eukaryotic (Määttä et al., 2009; Taskinen et al., 2013) and prokaryotic Avds (Avraham et al., 2015; Meir et al., 2009, 2012; Sardo et al., 2011) providing alternative protein scaffolds, more generally referred herein as Avd-Btn technology. A few natural bacterial Avds have a C-terminal extension acting as intrinsic ligand (Avraham et al., 2015; Leppiniemi et al., 2012; LeTrong et al., 2006; Meir and Livnah, 2010). These peptide ligands have the potential to be used as commercial tags *e.g.* Avi-tag (Meyer et al., 2006). Protein engineering through site-directed mutagenesis has been used to study the Btn-binding affinity of Avds (Chilkoti et al., 1995a, 1995b; Dixon and Kollman, 1999; Freitag et al., 1999; González et al., 1997, 1999; Hemminki, 1998; Laitinen et al., 1999, 2001, 2003; LeTrong et al., 2003; Marttila et al., 1998, 2000; Morag et al., 1996; Murakami et al., 2000; Nordlund et al., 2003; Pazy et al., 2001, 2003; Qureshi et al., 2001; Reznik et al., 1996, 1998; Sano and Cantor, 1995; Sano et al., 1995). Genetically engineered Avds were created to alter stability,

physicochemical and immunological properties, as well to change the ligand-binding affinity of selected monomer/s to either bind Btn with a lower affinity or bind ligands other than Btn *e.g.* peptide-tags, steroids; while maintaining the tight binding affinity of other monomers intact (Hytönen et al., 2005a, 2005b; Laitinen et al., 2006; Nordlund et al., 2004, 2005a; Riihimäki, 2011).

In this study, we solved the X-ray structure of core-bradavidin in complex with Btn, an artificially truncated form of wild-type (wt) bradavidin with an intrinsic C-terminal ligand 'Brad-tag' (Leppiniemi et al., 2012). We also solved the crystal structure of a steroid-binding Avd, sbAvd-2 (I117Y) in both the unliganded and progesterone-bound form. In addition, we built homology models of several artificial chicken Avd-based proteins – antidins – and performed computational docking studies with progesterone and Btn. All of these novel antidins provide the possibility to create engineered sc and dc Avds with up to four or two, respectively, Avd domains having ligand-binding affinities for non-Btn ligands. The Avds described in this study have potential for use in various applications in the field of bio(nano)technology and diagnostics. Furthermore, this study helps us to better understand the fine architecture of Avds and how the Avd scaffold could be further engineered towards improved binding preferences for non-Btn ligands of interest.

# 2. Review of literature

## 2.1 Chicken avidin

### 2.1.1 History of chicken avidin

The high affinity between chicken Avd and Btn was responsible for the early discovery of this glycoprotein when Eakin *et. al.* discovered that animals maintained on a strict egg white diet were deficient in Btn despite having the vitamin in their diet (Eakin et al., 1941; György and Rose, 1941). Apart from the use of Btn as a vitamin and nutrient (Sebrell and Harris, 1954), Btn is also involved in post-transcriptional events in Avd induction by progesterone (Tuohimaa et al., 1976). Chicken Avd was long regarded as a chicken oviduct and progesterone-specific protein (Kohler et al., 1968; O'Malley, 1967) but it was later seen that Avd can also be induced in a progesterone-independent environment in several other chicken tissues; for *e.g.*, during an inflammatory reaction caused by tissue trauma, pinching or heat injury, or by infection via microorganisms (Elo, 1980; Elo and Korpela, 1984; Elo et al., 1975, 1981, 1979a, 1979b; Heinonen and Tuohimaa, 1976; Heinonen et al., 1978; Korpela et al., 1983; Kulomaa, 1982; Niemelä et al., 1986; Nordback et al., 1981, 1982).

Once the role of Btn as a cofactor of enzymes was discovered (Wakil et al., 1958), the strong binding of Avd with Btn was utilized as a tool for studying the mode of action of Btn in carboxylation and transcarboxylation reactions (Finn et al., 1979; Hofmann et al., 1982). In fact, most of the research on Avds in the 1970's concentrated on the variety of application achieved by the Avd-Btn interaction, which is now known as Avd-Btn technology. These technologies are reviewed *e.g.* by (Airenne et al., 1999; Laitinen et al., 2007; Wilchek and Bayer, 1989, 1990); and include uses as bioaffinity sensors, for affinity targeting, pretargeting, drug delivery, and flow cytometry.

### 2.1.2 Distribution and function of chicken avidin

Chicken Avd was first discovered in hen egg-white (Eakin et al., 1941), followed by identification of Avds in the egg-white of turkey, duck, goose,

and in the egg jelly of frog (Hertz and Sebrell, 1942). Since then, the distribution of Avd in the egg-white of oviparous species has been observed and studied (Korpela et al., 1981). Due to the occurrence of Avd in the genital tracts and eggs of birds, and its regulation by steroids, an important role for Avd had been suggested in the physiology of avian reproduction (Hertz, 1946; Hertz and Sebrell, 1942; Korpela et al., 1981), but the exact role(s) of Avd in reproduction is still unresolved.

Chicken Avd could act to store the essential vitamin for use by the growing embryo. Although, the highest non-covalent binding observed in nature argues against this since the binding is essentially irreversible. The chicken egg white contains other Btn-binding Avd-like proteins with different affinities towards Btn, which could provide functions such as Btn storage and release, and other yet unknown functions (see section 2.4).

An anti-microbial function of high-affinity chicken Avd seems most likely, as inhibition of several microorganisms was demonstrated by Eakin *et. al.* and Green (Eakin et al., 1941; Green, 1975). The antimicrobial function of the bacterium *Streptomyces* sp. (known for producing a wide range of antibiotics) containing streptavidin, and the similarity of streptavidin and chicken Avd is already known (Green, 1975). The eggs of hens have multiple proteins acting to protect the egg from contamination, including lysozyme, conalbumin and riboflavin-binding protein, and it is not unreasonable to suggest that the extreme Btn-binding affinity of Avd plays a protective role, too. Chicken Avd could function to mop-up the free Btn in the egg white and hence help prevent infection of the egg by microorganisms, which the egg will encounter in the cloaca – where excretion also takes place and thus is highly contaminated – and in the environment after laying.

Apart from the excretory/genital tracts and eggs, chicken Avd was also found in the injured tissues of chicks (Eakin et al., 1940; György, 1939; György et al., 1941). Avd production was caused by the local muscular injury and was restricted to the inflammation site. This suggested a possible function of Avd during tissue inflammation of chickens (Elo, 1979; Elo and Korpela, 1984; Elo et al., 1981). Both chicken Avd and streptavidin, collectively called as (strept)avidin, have also been suggested to have a role

in the regulation of cell proliferation (Zerega et al., 2001). Bjavidin 1 and 2 from *Branchiostoma japonicum* have recently been shown to bind Btn but also recognize bacteria and enhance phagocytosis (Guo et al., 2017).

### 2.1.3 Structure of chicken avidin

Green and his coworkers studied the chicken Avd molecule through preliminary crystallographic analysis of Avd crystals (Green and Joynson, 1970) as well as electron-microscopic examination (Green et al., 1971) and concluded that chicken Avd tertiary structure has four subunits arranged in a two-fold symmetry; later proved by X-ray crystal structures (Livnah et al., 1993a; Pugliese et al., 1993, 1994).

Chicken Avd is a homotetrameric protein (Green and Toms, 1970) with each monomer made of eight antiparallel β-strands. The ligand-binding site is located on one end of each monomer, lined by polar and aromatic residues (Livnah et al., 1993a); each subunit has two cysteine residues forming a disulphide bridge that contributes to Avd stability (Green, 1975).

Chicken Avd is about 62 KDa in size. Chicken Avd is proteolytically cleaved releasing the N-terminal signal peptide of 24 amino acids and producing the mature protein of a 128 amino acids; chicken Avd is glycosylated at Asn17 (Gope et al., 1987; Green, 1975). The protein has an isoelectric point of pH 10.5. The dissociation constant, $K_d$, of chicken Avd from Btn, at a pH nefar 7, is ~$10^{-15}$ M. The strong avidity for Btn is the most characteristic property of the Avd protein. Avd is also highly thermostable, with the $T_m$ increasing from ~85°C in the absence of Btn to about 118°C when Btn is bound (Donovan and Ross, 1973; González et al., 1999; Nordlund et al., 2003).

The tetramers in Avd are arranged in a 1-4, 2-3 fashion (subunit numbering from (Livnah et al., 1993a)), as a dimer of dimers (Figure 1). Being a tetrameric protein, the monomers are held together by three forms of monomer-monomer interactions; subunit 1 - subunit 4 (IF1,4), subunit 1 - subunit 3 (IF1,3) and subunit 1 - subunit 2 (IF1,2) interface is stabilized by hydrogen bonds (H-bonds), hydrophobic bonds and van der Waals interactions (Livnah et al., 1993a), which contribute to the rigidity of the quaternary structure and directly to

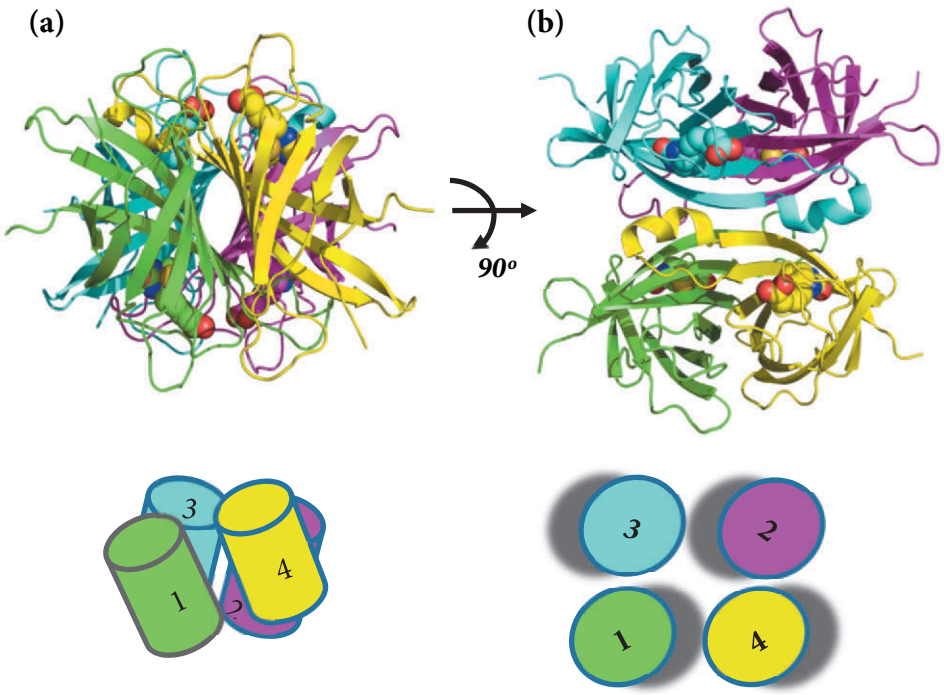


***Figure 1: Cartoon model of the chicken Avd [PDB: 1AVD] (Pugliese et al., 1993).*** *(a) tetrameric structure of chicken Avd (top) and a schematic representation (bottom) showing the arrangement of the subunits 1-4. (b) side view of the chicken Avd tetramer (left) and a top view of the tetramer (right). The monomers are arranged in a "1-4 and 2-3 scheme" similar to as described in Livnah et.al. 1993a. The four subunits are colored green (subunit 1), magenta (subunit 2), cyan (subunit 3) and yellow (subunit 4). The carbon atoms of the Btn ligand in each subunit are colored according to their subunit and are shown as spheres. Nitrogen atoms in Btn are colored blue, oxygen atoms red and sulphur atoms yellow. The figure scheme is adapted from Meir et al. (2009) Journal of molecular biology, 379–390.*

the Btn-binding mode of Avds (see below) (Livnah et al., 1993a). However, even slight changes in key amino acid residues at the interface are able to disrupt the tetrameric structure (Laitinen et al., 1999, 2001, 2003).

The largest contact surface area between the Avd subunits is formed by the IF1,4 interface (similar to IF2,3) with a surface area of 1951 Å$^2$ per monomer. IF1,4 forms a structural pair supported by a number of H-bonds and other weak interactions between them. The IF1,2 interface has a surface

area of 729 Å$^2$. IF1,3 has an area of 120 Å$^2$ making it the weakest monomer-monomer interface. The major interactions at this interface are polar and van der Waals interactions among hydrophobic residues Met96, Val115 and Ile117.

In the Btn-free apo form, water molecules partially occupy the chicken Avd binding site but are easily displaced by Btn. When Btn binds to Avd, the L3,4 loop (loop region connecting β-strand 3 and 4) adopts a closed conformation, acting as a lid to tightly seal Btn inside the binding pocket (Pugliese et al., 1994) Trp110 in the L7,8 loop from the neighboring subunit holds special importance during Btn binding because it seals off the binding site from one end, and forms a hydrophobic pocket with Phe79 and Trp97 from the original subunit, holding Btn in place (Livnah et al., 1993a).

The chicken Avd-Btn complex requires extreme environmental conditions for denaturation: it is stable over a wide range of pH and temperature, and is resistant to most proteases (Donovan and Ross, 1973; Green, 1963a). Chicken Avd, either in its apo form or bound with Btn, has use in various applications (See section 2.6), *e.g.* affinity chromatography, diagnostics, immunoassays and drug delivery (Wilchek and Bayer, 1989).

## 2.2 Streptavidin

Streptavidin was first isolated in culture media from several species of *Streptomyces* (Chaiet and Wolf, 1964; Tausig and Wolf, 1964). The chemical nature, structure and Btn-binding properties of streptavidin had been observed to be similar to chicken Avd (Chaiet and Wolf, 1964; Tausig and Wolf, 1964), with the $K_d \sim 10^{-14}$ M for the streptavidin-Btn complex (Green, 1990). The thermostablility of streptavidin with attached Btn is ~112°C and *sans* Btn is ~84°C (González et al., 1999).

Native full-length streptavidin contains 159 amino acids and is processed at the N- and C-termini to give what is called core-streptavidin, having typically 127 amino acids (Pähler et al., 1987). The truncated core-streptavidin has higher solubility in water and has been shown to have better Btn-binding properties than wild-type (wt) streptavidin (Green, 1990). Core-streptavidin also lacks cysteine and methionine residues, but has six tryptophan residues per subunit (Chaiet and Wolf, 1964). The lack

of cysteine residues means that, unlike chicken Avd, streptavidin has no disulphide bridge (Livnah et al., 1993a).

Soon after solving the crystal structure of streptavidin (Weber et al., 1989), it was suggested that streptavidin is structurally similar to lipocalins, another member of the calycin superfamily (Flower, 1993) – lipocalins also consists of eight anti-parallel β sheets, with a ligand-binding site in the middle of the β-barrel structure. In comparison to streptavidin, the loop architecture of lipocalins is different, the N-terminal sequence seals the β-barrel and a characteristic α-helix after the β8 strand is present on the C-terminal end (Flower, 1993).

Within the Avd family of proteins, X-ray analyses have shown that the even though chicken Avd and streptavidin are only about 35% identical in amino acid sequence, the folds of both Avds are conserved, and so are most of the residues important for binding Btn (Bayer and Wilchek, 1990). The importance of tryptophan in Btn binding was stressed by Green (Green, 1975), and confirmed by site-directed mutagenesis on streptavidin (Chilkoti et al., 1995a; Laitinen et al., 1999; Sano and Cantor, 1995; Wu and Wong, 2005). Other key residues were similarly shown to be important for Btn binding, including tyrosine (Tyr43 in streptavidin) (Gitlin et al., 1989, 1990; Hiller et al., 1991) and lysine (Lys45, Lys94, Lys111 in streptavidin) (Gitlin et al., 1987). Streptavidin also differs in sequence at the binding site with respect to chicken Avd: Phe79 in chicken Avd is replaced by Trp92 in streptavidin while Phe72 in chicken Avd appears not to have a structural counterpart in streptavidin. In addition, the L3,4 loop in streptavidin is shorter by four residues in comparison with chicken Avd (Stenkamp et al., 1997).

The major subunit interfaces in streptavidin are similar to those in chicken Avd. However, in the case of IF1,3, Gln97 and His127 in streptavidin replace Met96 and Ile117 in chicken Avd; the side chain of His127 from two subunits participate in π-π stacking with each other at the interface, contributing to streptavidin's stability (Livnah et al., 1993a).

Core-streptavidin, wt streptavidin and a Btn-conjugated form are all used in various applications such as for immobilizing agents, flow cytometry,

histochemistry, and bioaffinity sensors ((Wilchek and Bayer, 1989) and references therein).

## 2.3 (Strept)avidin-biotin binding

Chicken Avd has four binding sites for Btn, one in each subunit (Chignell et al., 1975; Green, 1966). In addition to hydrophobic interactions by tryptophan and phenylalanine residues (Chilkoti et al., 1995a), the ureido ring of Btn in chicken Avd is involved in five crucial hydrogen bonding interactions: with Asn12, Ser16, Tyr33, Thr35 and Asn118. In addition, the sulfur atom of Btn interacts with Thr77, and the two carboxylate oxygen atoms of the valeric acid tail of Btn can H-bond with Thr38, Ala39, Thr40, Ser73, Ser75 (Livnah et al., 1993a; Pugliese et al., 1993) making a total of up to eleven hydrogen bonds for the Avd-Btn complex.

In streptavidin, a similar network of H-bonds is observed. The ureido ring forms three H-bonds: with Asn23, Ser27 and Tyr43. The ring nitrogen atoms N1′ and N3′ respectively H-bond with Asp128 and Ser45. The sulfur atom of Btn forms an H-bond with Thr90, and the carboxylate oxygen atoms of the valeric acid tail form two H-bonds: one with the main-chain nitrogen atom of Asn49 and another with the side-chain hydroxyl group of Ser88 (Livnah et al., 1993a; Weber et al., 1989) making a total of eight H-bonds for the streptavidin-Btn complex. The existence of fewer H-bonds, together with a lack of one hydrophobic interaction (equivalent to Phe72 in chicken Avd), leads to 100-fold lower affinity for Btn by streptavidin than is seen for chicken Avd (Figure 2).

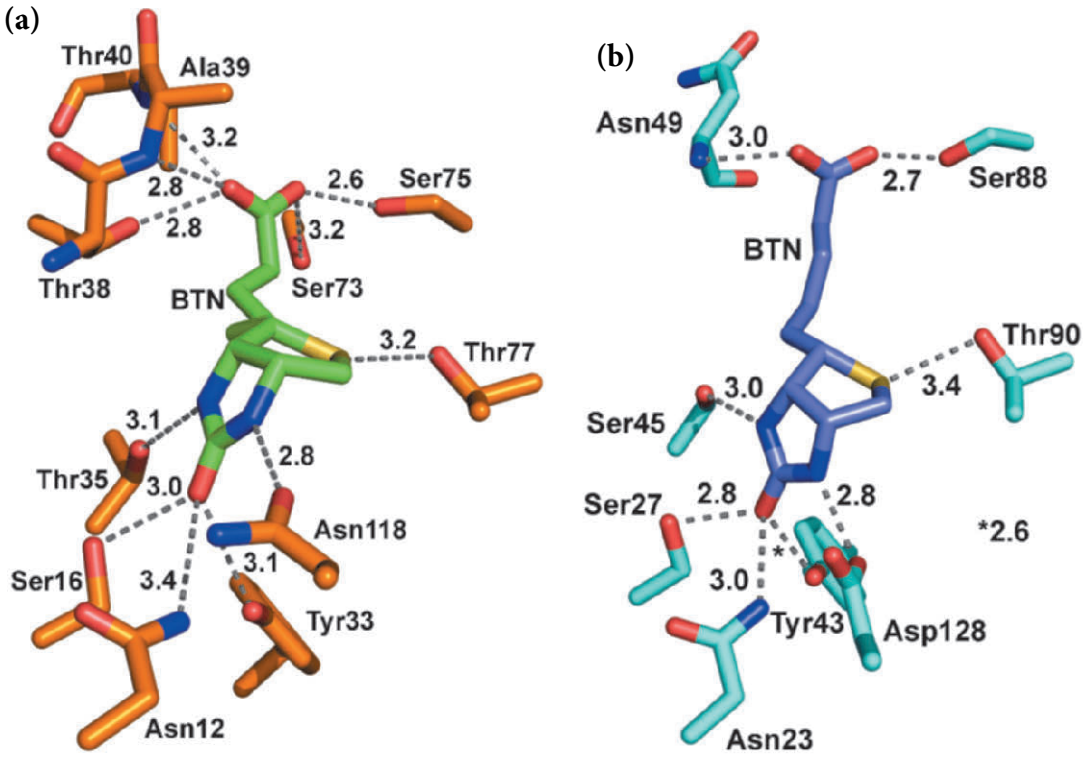


***Figure 2: Hydrogen bonding network in (a) chicken Avd [PDB: 1AVD] (Pugliese et al., 1993) and (b) streptavidin [PDB: 1STP] (Livnah et al., 1993a; Weber et al., 1989).*** *(a) Side chains of residues forming H-bonds with Btn (green carbon atoms) in chicken Avd [PDB: 1AVD] (orange carbon atoms) and (b) with Btn (purple carbon atoms) in streptavidin [PDB:1STP] (cyan carbon atoms) are shown as thick sticks. Nitrogen atoms are colored blue, oxygen atoms red and sulfur yellow. The H-bonds are drawn as grey dashed lines and the distances are in Ångströms.*

## 2.4 Other natural avidins and biotin-binding proteins

Owing to their early discovery, the best-studied Avds are chicken (*Gallus gallus*) Avd and the bacterial streptavidin from *Streptomyces avidinii*. Apart from these two well-studied Avds, there are many other Avds from different organisms whose sequences have been reported and some have been expressed and characterized in detail, including structural studies.

Avds from several other eukaryotic species (see (Tiwari, 2015)), such as Ostrich (*Struthio camelus australis*) (Zhang et al., 2014), American alligator (*Alligator mississippiensis*) (St John et al., 2012); fungal Avds like lentiavidins from *Lentinula edodes* (Takakura et al., 2016); bacterial Avds such as burkavidin from *Burkholderia pseudomallei* (Sardo et al., 2011), as well as *Mycobacterium thermoresistible* and the recently discovered BjAvidin 1 and 2 from the invertebrate lancelet (amphioxus;*Branchiostoma japonicum*) (Guo et al., 2017) have been described.

Examples of different Avds with solved three-dimensional (3D) structures include: the eukaryotic Avds zebavidin [PDB: (4BJ8)] from zebrafish (*Danio rerio*) (Taskinen et al., 2013), xenavidin [PDB: 2UYW] from frogs (*Xenopus tropicalis*)(Määttä et al., 2009); the fungal Avds tamavidin 1 and 2 [PDB: 2ZSC] from mushrooms (*Pleurotus cornocopiae*) (Takakura et al., 2009); and the bacterial Avds bradavidin [PDB: 2Y32] (Leppiniemi et al., 2012) and bradavidin II [PDB: 4GGR] (Leppiniemi et al., 2013) from *Bradyrhizobium japonicum* (Delamuta et al., 2013; Helppolainen et al., 2008; Nordlund et al., 2005b), dimeric rhizavidin from *Rhizobium etli* [PDB: 3EW1](Helppolainen et al., 2007; Meir et al., 2009), shwanavidin [PDB: 3SZJ] from the bacterium *Shewanella denitrificans* (Meir et al., 2012) and hoefavidin [PDB: 4Z6J] from *Hoeflea phototropica* (Avraham et al., 2015) (Table 1).

*Table 1: Examples of experimentally determined 3D Avd structures from the PDB*

| **Protein** | **Organism Name** | **PDB ID** | **Oligomeric state** | **Reference** |
|---|---|---|---|---|
| Chicken avidin | *Gallus gallus* | 1AVD | Tetrameric | (Pugliese et al., 1993) |
| Streptavidin | *Streptomyces avidinii* | 2BC3 (Full-length)<br>3RY1 (Core) | Tetrameric<br>Tetrameric | (LeTrong et al., 2006)<br>(LeTrong et al., 2011) |
| Xenavidin | *Xenopus tropicalis (Western clawed frog)* | 2UYW | Tetrameric | (Määttä et al., 2009) |
| Zebavidin | *Danio rerio (zebrafish)* | 4BJ8 | Tetrameric | (Taskinen et al., 2013) |
| Bradavidin | *Bradyrhizobium diazoefficiens* | 2Y32 | Tetrameric | (Leppiniemi et al., 2012) |
| Tamavidin | *Pleurotus cornucopiae (Tamogitake mushroom)* | 2ZSC | Tetrameric | (Takakura et al., 2009) |
| Bradavidin II | *Bradyrhizobium diazoefficiens* | 4GGR | Uncertain (highly oligomeric) | (Leppiniemi et al., 2013) |
| Rhizavidin | *Rhizobium etli* | 3EW1 | Dimeric | (Meir et al., 2009) |
| Shwanavidin | *Shwanella denitrificans* | 3SZJ | Dimeric | (Meir et al., 2012) |
| Hoefavidin | *Hoeflea phototrophica* | 4Z6J | Dimeric | (Avraham et al., 2015) |

The essential Btn-binding residues are partly conserved in Avds, depending on the oligomeric state (tetrameric/dimeric) and species (eukaryotic/prokaryotic). For example, through sequence alignment of identified Avd sequences, it was observed that all tetrameric Avds have a tryptophan residue identical to Trp110 in chicken Avd. It is one of the most critical amino acids needed for Btn binding, as it seals the binding pocket from one side (Chilkoti et al., 1995a; Laitinen et al., 1999; Sano and Cantor, 1995), as well as ensuring the stability of the tetramer by contributing to IF1,3 subunit interactions.

In addition to chicken Avd, the chicken genome contains other Btn binding and Avd-like proteins, namely: biotin-binding proteins (BBPs) (Bush et al., 1988; Meslar et al., 1978; White et al., 1976) and avidin-related proteins (AVRs) (Ahlroth et al., 2000; Hytönen, 2005; Keinänen et al., 1988, 1994; Wallén et al., 1995). Even though the overall tetrameric structure is conserved, BBPs and AVRs presumably differ in their functional roles in chicken. In general, BBPs and AVRs have lower affinity towards Btn in comparison to chicken Avd and even recognize ligands other than Btn; BBP-A, for example, D-biotin D-sulfoxide (BSO) (Hytönen et al., 2007). Their physiochemical properties, *e.g.* isoelectric point and thermal stability, also vary considerably from chicken Avd. Examples of a few natural Avds, BBPs, and AVR proteins are discussed in detail below.

**2.4.1 Biotin-binding proteins (BBPs) –** Around the same time as chicken egg white Avd was discovered, the egg yolk was thought to contain another macromolecule forming a complex with Btn (György and Rose, 1941). However, identification of what turned out to be two biotin-binding proteins – BBP-I and BBP-II – was made many years later (Bush et al., 1988; Meslar et al., 1978; Subramanian and Adiga, 1995; White and Whitehead, 1987; White et al., 1976). Due to their lower affinity for Btn in comparison to chicken Avds, BBPs were thought to serve as carrier proteins, which deliver Btn to the developing chick embryo, as they could release Btn as and when required (White and Whitehead, 1987).

In comparison to BBP-II (Bush et al., 1988; Subramanian and Adiga, 1995; White and Whitehead, 1987), BBP-I had higher affinity for Btn ($K_d$~$10^{-12}$ M) as well as a thermal stability similar to chicken Avd (Meslar et al., 1978;

White et al., 1976). It is still not known with certainty whether the cDNAs encoding two other reported chicken Avds, BBP-A and BBP-B (Niskanen et al., 2005), correspond to BBP-I or BBP-II.

The crystal structure of BBP-A is tetrameric [PDB: 2C1Q] (Hytönen et al., 2007) (Figure 3a), but is less stable ($T_m$ of 103.4°C in the presence of Btn) than chicken Avd. The reason for this may be the higher positive surface charge and 10% larger solvent accessible surface area, along with higher β-barrel flexibility of BBP-A [PDB: 2C1Q] in comparison to chicken Avd-Btn complex [PDB: 2AVI]. The affinity of BBP-A towards Btn ($K_d$~$10^{-13}$ M) is quite similar to that of streptavidin, and interestingly, BBP-A binds D-biotin D-sulfoxide (BSO) tighter ($K_{diss}$~1.3 x $10^{-4}$ $s^{-1}$) than Btn ($K_{diss}$~5.4 x $10^{-4}$ $s^{-1}$) (Hytönen et al., 2007).

**2.4.2 Avidin-related proteins (AVRs) –** Avidin-related genes were first cloned by Keinänen in 1988 with the discovery of AVR1, AVR2 and AVR3 (Keinänen et al., 1988). AVR4 and AVR5 were cloned in 1994 (Keinänen et al., 1994), and AVR6 and AVR7 in 2000 (Ahlroth et al., 2000). The number of AVR genes varies among individual chicken cells, possibly because of frequent recombination inside the gene family (Ahlroth et al., 2001).

The discovery of AVRs led to the possibility that the chicken genome may have more than one functional gene for Avd, but this does not appear to be the case. Out of the seventeen residues that interact with Btn in chicken Avd, between twelve and fourteen are conserved in AVR genes (Keinänen et al., 1994; Laitinen et al., 2002). Furthermore, in comparison to chicken Avd (Laitinen et al., 2002), the AVRs have distinctly different properties: Btn-binding affinities range from the Avd-like tight affinity of AVR4 ($K_d$ ~$10^{-14}$ M) (Eisenberg-Domovich et al., 2005; Hytönen et al., 2004) to the affinity of AVR2 ($K_d$ =5.2x$10^{-8}$ M) (Laitinen et al., 2002). In addition, AVRs can be even more thermostable than chicken Avd ($T_m$ ~85°C for the apo protein and ~118°C for the Btn-bound form), *e.g.* AVR4 ($T_m$ ~106°C for apo protein and ~125°C for the Btn-bound form). The increased thermostability of AVR4 was suggested to be due to Tyr115 at the IF1,3 interface and the shorter L4,5-loop (Hytönen et al., 2004) (Figure 3b).

**2.4.3 Rhizavidin** – Rhizavidin was isolated from the nitrogen-fixing bacterium *Rhizobium etli* found in the root nodules of leguminous plants

(Helppolainen et al., 2007) and was the first natural dimeric Avd structure solved from the Avd family (Meir et al., 2009) [PDB: 3EW1]. The two monomers in rhizavidin form a structural pair similarly to the monomers forming the IF1,4 interface in chicken Avd (Meir et al., 2009) (Figure 3c). Even though rhizavidin is dimeric in solution, it formed a hexameric assembly (trimer of dimers) in the asymmetric unit of the crystal. In the hexameric assembly, the C-terminal end (residues 132-135) from one rhizavidin monomer binds to the Btn-binding site of the adjacent dimer in the hexamer and *vice versa* (Meir and Livnah, 2010).

The L7,8 loop of rhizavidin, which is shorter in comparison to most other Avds, has a unique Gly-Gly-Ser-Gly sequence, resulting in a different loop conformation. Rhizavidin also has a disulphide bridge (a structural feature seen in the dimeric Avds shwanavidin (Meir et al., 2012) and hoefavidin (Avraham et al., 2015)), connecting the L3,4 and L5,6 loop, hence forming a different conformation of the L3,4 loop in comparison to chicken Avd. Unlike for tetrameric Avds, in dimeric rhizavidin there is no analogous tryptophan to Trp110 present in chicken Avd, hence the disulphide bridge is important in providing rigidity to the structure as well as to the ligand-binding site.

The disulphide bridge in rhizavidin also aids in Btn-binding, but the affinity of rhizavidin for Btn is still weaker than for tetrameric Avds ($K_d$ ~$10^{-9}$ M) (Helppolainen, 2009). Livnah and coworkers have described the importance of the L3,4 loop conformations and how it affects the ligand binding of chicken Avd and streptavidin (Livnah et al., 1993a; Pazy et al., 2002). In rhizavidin, the difference in the L3,4 loop conformation together with the exposure of the binding site due to the absence of Trp110 can be a possible explanation for the weaker Btn-binding affinity of rhizavidin. The $T_m$ of apo-rhizavidin is 75˚C, which is close to the $T_m$ of apo-streptavidin, but the $T_m$ of Btn-bound rhizavidin is only 100.5°C, significantly lower than Btn-bound streptavidin with $T_m$ of 112°C (González et al., 1999; Helppolainen et al., 2007).

**2.4.4 Wild-type bradavidin** – Bradavidin was identified in *Bradyrhizobium diazoefficiens* (Delamuta et al., 2013; Nordlund et al., 2005b), a nitrogen-fixing bacteria present in the root nodules of soybean (Nordlund et al., 2005b). The crystal structure of this homotetrameric

protein was solved by Leppiniemi *et al.* in 2012 [PDB:2Y32] (Leppiniemi et al., 2012).

The X-ray structure of wt bradavidin revealed that the C-terminal tail acts as an intrinsic intersubunit ligand bound to the Btn-binding pocket of an adjacent subunit, *e.g.* the binding pocket of subunit 1 is occupied by the C-terminal end of subunit 3 and *vice versa* (Figure 3d). This bound peptide GSEKLSNTKK, was named the "Brad-tag" (Leppiniemi et al., 2012) and three residues – Glu131, Lys132 and Leu133 – occupy the site equivalent to where Btn binds in chicken Avd [PDB:1AVD]. The homotetrameric wt bradavidin has a disulphide bridge stabilizing the L3,4 – L5,6 loop, which is typical of the identified dimeric Avds (see section 2.4.3). To accommodate the Brad-tag, the ligand-binding pocket widens, the disulphide bridge conformation alters and the L3,4 loop stays in the 'open' conformation (Leppiniemi et al., 2012). In addition to wt bradavidin with 138 amino acid residues, an engineered C-terminally truncated "core-bradavidin" with 118 residues – lacking residues Gly114-Lys138 – was also produced (Nordlund et al., 2005b).

Bradavidin shares less than 30% sequence identity with chicken Avd and streptavidin, and is immunologically different, too. Wild-type bradavidin has an acidic pI of 6.3 and core-bradavidin has a pI of 4.1. The Btn-binding affinity of wt bradavidin is also weaker ($K_d$~$10^{-7}$ M) than that of core-bradavidin ($K_d$~$10^{-9}$ M), indicating that the Brad-tag competes and interferes with Btn binding to wt bradavidin. The $T_m$ of wt bradavidin *sans* Btn is 96°C, whereas in the presence of Btn the $T_m$ increases to 101°C; this difference and magnitude of the $T_m$ values are not as pronounced as for other tetrameric Avds.

Bradavidin is the first Avd seen to have an intrinsic *intersubunit* ligand, naturally bound to the Btn-binding site. In contrast, wt streptavidin binds the C-terminal Strep-tag sequence within the Btn binding site of the same subunit (Figure 4), hence making it an *intrasubunit* ligand (LeTrong et al., 2006). The Brad-tag is also found in rhodavidin from *Rhodopseudomonas palustris* (Larimer et al., 2004) and a few other *Bradyrhizobium* species.

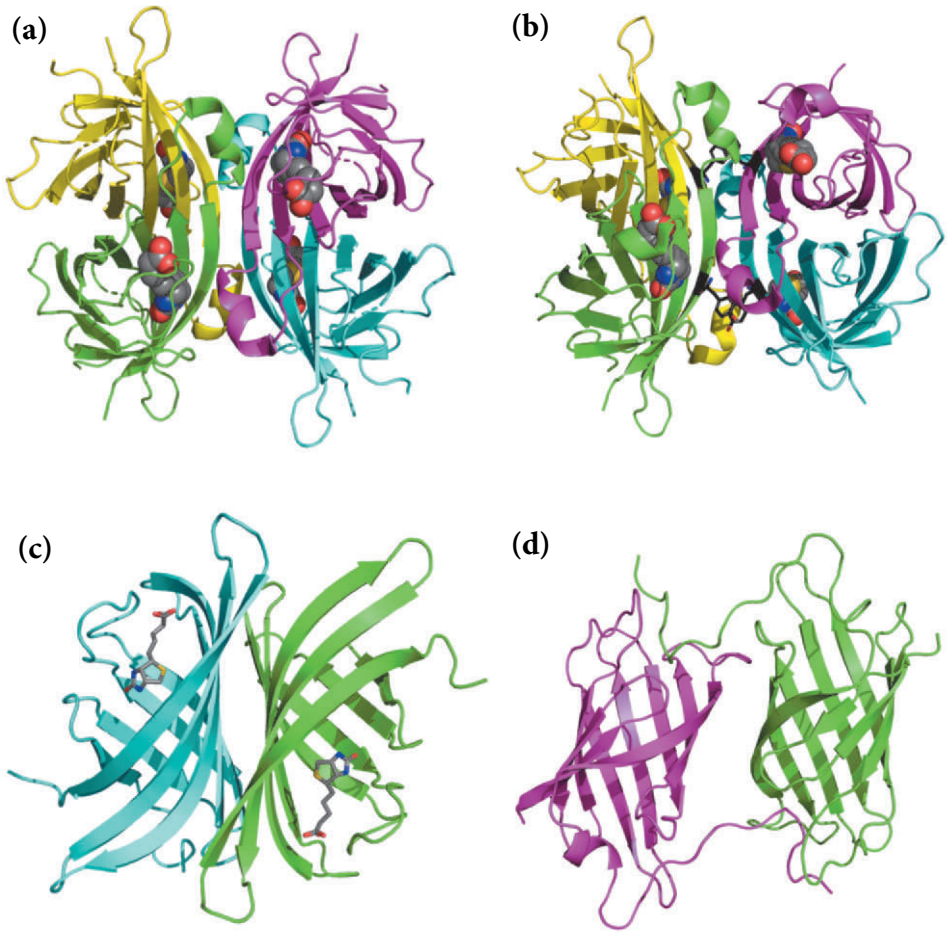


***Figure 3: Cartoon model of (a) tetrameric BBP-A [PDB:2C1Q], (b) tetrameric AVR4 [PDB: 1Y55], (c) dimeric rhizavidin [PDB:3EW2] and (d) subunit 1 and 3 from tetrameric wt bradavidin [PDB:2Y32].*** *(a) Subunits 1-4 in BBP-A are colored as in Figure 1. Btn is shown in spheres (grey carbon atoms). (b) Subunits 1-4 and Btn colored as in Figure 1. Additionally, Tyr115, known to increase thermostability of AVR4, is shown as sticks (black). (c) Subunit 1 (cyan) and subunit 2 (green) are shown in the dimeric rhizavidin. Btn is shown as sticks (grey). (d) Subunits 1 (magenta) and 3 (green) are shown from wt bradavidin. Nitrogen atoms in all the panels are colored blue, oxygen atoms red and sulfur yellow.*

The potential of the Brad-tag for use in Avd-Btn technology was tested by creating Brad-tag–EGFP (enhanced green fluorescent protein) fusion proteins, with and without a poly-histidine tag. In short, agarose beads were

conjugated to core-bradavidin in order to purify Brad-tagged EGFP from other proteins in the cell lysate. The Brad-tag is specific for the bradavidin-binding site and binds to core-bradavidin with micromolar affinity; the Brad-tag did not bind to chicken Avd, streptavidin or rhizavidin (Leppiniemi et al., 2012).

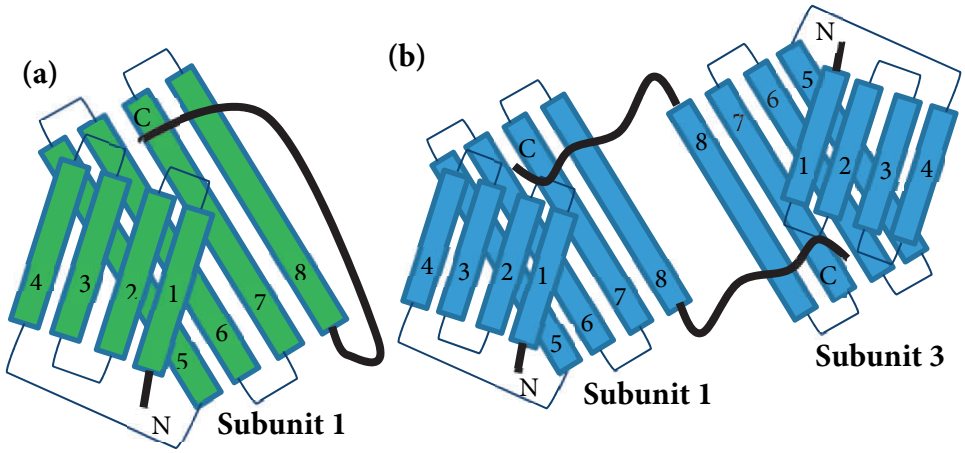


***Figure 4: Schematic representation of intrinsic peptide ligand binding in (a) streptavidin [PDB: 2BC3] and (b) wt bradavidin [PDB: 2Y32].*** *(a) Subunit 1 of streptavidin (green) is shown with the C-terminal acting as a intrasubunit ligand binding to the Btn-binding site. (b) In contrast, subunit 1 and 3 of wt bradavidin (blue) are shown where the C-terminal acts as a intersubunit ligand binding to the Btn-binding site of the neighboring subunit. The N and C terminals of both the Avds are shown in black.*

## 2.5 Ligands of avidin

### 2.5.1 Biotin and its analogues

The natural ligand of Avds is Btn. The importance of Btn for various biochemical and cellular processes was already noted quite early (György, 1939), whereas the structure of Btn itself was determined much later (DeTitta et al., 1976; Trotter and Hamilton, 1966). Enzyme-bound Btn functions as a carrier of an activated carboxyl group in carboxylation, decarboxylation and transcarboxylation reactions (Chapman-Smith and Cronan Jr, 1999; McMahon, 2002; Wakil et al., 1958). Btn is also known to

regulate the expression of various genes, *e.g.* glucokinase (Chauhan and Dakshinamurti, 1991; Dakshinamurti and Litvak, 1970), Btn-dependent carboxylases and holocarboxylase synthetase (Rodríguez-Meléndez et al., 2001; Solórzano-Vargas et al., 2002), Btn-transporters (Crisp et al., 2004; Manthey et al., 2002), cytokines (Manthey et al., 2002; Rodríguez-Meléndez et al., 2003; Zempleni et al., 2001) and oncogenes (Pacheco-Alvarez et al., 2002). Deficiency of Btn in humans is known to cause a decline in carboxylase activity (Manthey et al., 2002) leading to hypoglycemia and ketosis due to a non-functional enzyme, pyruvate carboxylase (Bender, 1999). The functions of other enzymes, such as acetyl-CoA carboxylase (Kopinski et al., 1989), propionyl-CoA carboxylase (Bender, 1999) and methylcrotonyl-CoA carboxylase (Mock and Stadler, 1997; Mock et al., 1997), are also affected due to a lack of Btn. The specific mechanism of Avd-Btn binding is described in section 2.3.

**2.5.1.1 Biotin derivatives** – Even though Btn derivatives bind to the Avd ligand-binding site with a lower affinity than Btn, derivatives have played an important role in (strept)avidin-Btn technology. For example, Btn derivatives with alkyl and polyethylene glycol (PEG) chains coupled with DOTA (2,2′,2″,2‴-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid) have been used to detect pancreatic islet of Langerhans coated with Avd in a liver transplantation model (Blom et al., 2009). Photobiotin, another Btn derivative, can be linked to amino groups by photoactivation. This molecule can be further used to label DNA and RNA (Forster et al., 1985), and for covalent immobilization of various macromolecules at defined locations on nitrocellulose and polystyrene surfaces (Hengsakul and Cass, 1996).

The binding of different derivatives to Avd suggests that the ureido ring of Btn is of great importance for tight binding ((Green, 1975) and references therein) – compounds that have modifications of the ureido ring bound less firmly to the chicken Avd-binding site than derivatives without modifications to the ring (Green, 1963b, 1966). A summary of these derivatives can be found in (Green, 1975). Overall, it seems that every atom in the Btn molecule contributes to the interaction with Avd, leading to the maximal affinity observed for noncovalent binding in nature.

### 2.5.2 Other ligands of avidin

**2.5.2.1 Azo compounds** – Apart from Btn and its derivatives, some other small molecules such as azo derivatives (Repo et al., 2006), having little or no structural similarity to Btn, are also bound by Avds (Green, 1975). The binding affinities of these compounds are considerably lower in comparison to Btn, *e.g.* $K_d$~1 $\times 10^{-4}$ M for streptavidin (Green, 1990), and they are easily displaced in solution. For example, the displacement of an azo compound like 4-hydroxyazobenzene-2-carboxylic acid (HABA) by Btn has been used to quantitatively measure Btn-binding activity through spectrophotometric techniques developed by Green (Green, 1965). The affinity of chicken Avd ($K_d$~7.9 $\times 10^{-6}$ M for HABA (Määttä et al., 2008)) has been modified to improve the affinity for HABA. The N118 residue of Avd, which did not make a favorable interaction with HABA in the HABA-Avd complex (Livnah et al., 1993b), was mutated to methionine leading to a $K_d$ of 5.2 $\times 10^{-6}$ M; but the circular permutant cpAvd4→3 with this mutation had even higher HABA-binding affinity ($1 \times 10^{-6}$ M), while the Btn-binding affinity decreased to $2.4 \times 10^{-7}$ M (Määttä et al., 2008). A decrease in affinity for Btn as a result of mutagenesis of the corresponding site in streptavidin had already shown similar results (Qureshi et al., 2001).

**2.5.2.2 Peptide ligands** – Out of the identified natural bacterial Avds, a few have intrinsic peptides that bind to the Btn binding site: *e.g.*, streptavidin (LeTrong et al., 2006), wt bradavidin (Leppiniemi et al., 2012) and hoefavidin (Avraham et al., 2015) (some examples are listed in Table 2). These peptide ligands have a lower binding affinity to Avds in comparison to Btn, but they have been instrumental for advances in the Avd-Btn technology through the development of commercial tags: Avi- and AviD-tag (Gaj et al., 2007; Meyer et al., 2006), Strep-tag (a nine L-amino acid peptide which has affinity towards streptavidin) (Schmidt et al., 1996), Strep-tag II (an eight amino acid peptide) (Korndörfer and Skerra, 2002; Schmidt et al., 1996; Skerra and Schmidt, 2000, 1999; Voss and Skerra, 1997), Nano-tag15 and Nano-tag9 (a fifteen and nine amino acid long peptide with streptavidin affinity, respectively)(Lamla and Erdmann, 2004; Perbandt et al., 2007) and SBP-tag (a thirty-eight residue long peptide with affinity to streptavidin)(Keefe et al., 2001). These tags are used in various

applications, for example, purification of fusion proteins in one-step procedure (Cho et al., 2000; Keefe et al., 2001; Lamla and Erdmann, 2004).

*Table 2: Examples of peptide ligands of Avds*

| **Tag** | **Sequence** | **Receptor** | **PDB ID** | **References** |
|---|---|---|---|---|
| Avi-tag | DRATPY | Avidin, NeutrAvidin | Not Available (N.A.) | (Meyer et al., 2006) |
| AviD-tag | DRATPY-spacer-DRATPY | Avidin, NeutrAvidin | N.A. | (Gaj et al., 2007) |
| Nano-tag$_{15}$ | formyl-MDVEAW LGARVPLVET | Streptavidin | N.A. | (Lamla and Erdmann, 2004) |
| Nano-tag$_{9}$ | formyl-MDVEAWL GAR | Streptavidin | N.A. | (Lamla and Erdmann, 2004) |
| Nano-tag | formyl-MDVEAWL | Streptavidin | 2G5L | (Perbandt et al., 2007) |
| Strep-tag | AWRHPQFGG | Streptavidin | 1RST | (Schmidt et al., 1996) |
| Strep-tag II | WSHPQFEK | Streptavidin; SA mutant 1; SA mutant 2 | 1RSU; 1KL3; 1KL5 | (Schmidt et al., 1996) (Korndörfer and Skerra, 2002) (Skerra and Schmidt, 1999) |
| SBP-tag | MDEKTTGWRGGH VVEGLAGELEQLR ARLEHHPQGQREP | Streptavidin | 4JO6 | (Keefe et al., 2001) (Barrette-Ng et al., 2013) |
| Brad-tag | GSEKLSNTKK | Bradavidin | 2Y32 | (Leppiniemi et al., 2012) |
| Hoef-pep Form A | SVATVSESLLTE | Hoefavidin | 4Z2O | (Avraham et al., 2015) |

**2.5.3 Steroids** – Apart from the above mentioned ligands, in recent years, through engineering and mutagenesis of Avds, it has been possible to shift the ligand binding preference of some Avds towards non-natural ligands, *e.g.* steroids, for which chicken Avd possesses no natural affinity (Riihimäki et al., 2011a) (see the graphical abstract, publication I).

Steroids are a group of compounds containing a tetracyclic hydrocarbon sterane (Moss, 1989; Steroids, 2017). The word steroid is also used as a collective term for sterols, bile acids and sex hormones (Steroids, 2017). Steroids contain methyl groups at carbon atoms C10 and C13, and additional functional groups at C3 and C17 depending on the type of steroid (Moss, 1989) (Figure 5).

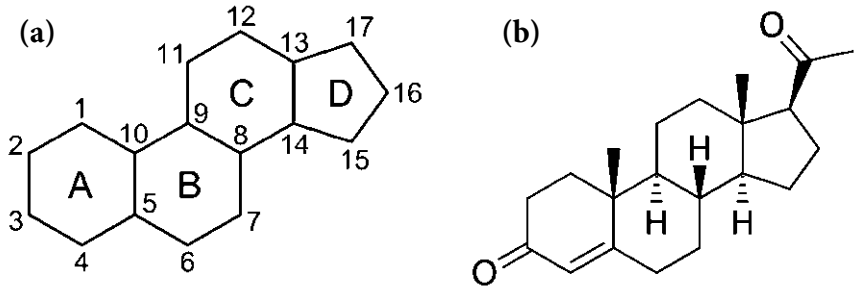


***Figure 5: 2D representation of (a) the steroid skeleton and (b) progesterone molecule.*** *(a) Tetracyclic steroid skeleton that forms the backbone of steroids. The four rings are marked in black and the carbons are numbered in red. (b) The skeleton of the progesterone molecule for comparison. It contains a methyl group at position C10 and C13 and two additional functional groups at C3 and C17, respectively.*

Cholic acid, a primary bile acid for humans, is produced in the liver and synthesized from cholesterol. Bile acids in general are essential in dietary lipid absorption and cholesterol catabolism. They are also essential in activating cell-signaling pathways (Hylemon et al., 2009), regulating glucose and lipid metabolism (Li and Chiang, 2011), and preventing obesity and resistance to insulin in humans (Watanabe et al., 2006).

Progesterone and testosterone are steroid sex hormones. Progesterone belongs to the category of progestogen sex hormones. It is essential in the female body, having a key role in pregnancy and menstruation (King and

Brucker, 2010). Apart from its role as a sex hormone, progesterone is essential in maintaining healthy brain activity (Roof and Hall, 2000) and skin (Raine-Fenning et al., 2003) in women. Testosterone belongs to the group of androgen sexual hormones and is essential for the development of male reproductive organs (Mooradian et al., 1987). It is also involved in maintenance of general well-being of the body. Both progesterone and testosterone are used in hormone replacement therapy (Bassil et al., 2009).

Cortisol is one of the major corticosteroid (Steroids, 2017). In vertebrates, it is important for controlling stress responses, immune responses and metabolism (Charmandari et al., 2005). Cortisol (hydrocortisone) is also used as an anti-inflammatory drug and for suppressing extreme allergic reactions (Elenkov, 2004).

## 2.6 (Strept)avidin-biotin technology

The isolation and identification of Btn was clearly associated with the discovery of chicken Avd (Eakin et al., 1941). The extremely high affinity with which Btn binds to chicken Avd and streptavidin ((strept)avidin) has provided the basis for the widely-used technology called (strept)avidin–Btn technology. Since its inception, the (strept)avidin–Btn technology has led to major improvements in the efficiency and effectiveness of several biochemical and biotechnological techniques and formed the basis of several new technologies in various fields of biology and medical diagnostics.

i) The major advantages of the strept(avidin)–Btn binding, which forms the underlying principle of this technology, adapted from (Wilchek and Bayer, 1990), are listed below:

a) The high affinity and stability of the strept(avidin)–Btn complex ensures tight binding between molecules and/or materials in which one of a pair is biotinylated and the other is coupled to strept(avidin) (Diamandis and Christopoulos, 1991).

b) Btn is readily attached to most targets without altering their original function.

c) The Avd-Btn system is extremely versatile in the choice of target-pairs it can be applied to.

d) A wide range of different biotinylating reagents and Avd–containing probes are available from various commercial sources (Wilchek and Bayer, 1990).

ii) A few applications that implement the strept(avidin)–Btn technology are listed below ((Airenne et al., 1999; Laitinen et al., 2007; Wilchek and Bayer, 1989, 1990):

a) Isolation of Btn-tagged macromolecules and compounds is efficient and effective via affinity chromatography, *e.g.* isolation of biotinylated enzymes (Berger and Wood, 1975; Bodanszky and Bodanszky, 1970; Landman and Dakshinamurti, 1973; Rylatt et al., 1977).

b) Biotinylated antibodies are used for visualization and localization of many different compounds in affinity cytochemistry, immunoassays and immunohistochemistry.

c) For study of the distribution, structure and function of cell surface molecules.

d) Quantitative assays in the presence or absence of the corresponding antibody for a given molecule (Wilchek and Bayer, 1989, 1990).

e) End-point markers in the study of the mode of action of steroid hormones, *e.g.* progesterone (Hertz et al., 1944; Kohler et al., 1968; O'Malley, 1967).

f) Targeting and accumulating biotinylated antibodies and therapeutic molecules inside tumor cells (pretargeting) (Jain and Cheng, 2017; Lesch et al., 2010).

g) Biotinylated organometals attached to strept(avidin) to develop artificial metalloenzymes for *e.g.* hydrogenation (Heinisch and Ward, 2016).

In order to exploit strept(avidin)–Btn binding in an even wider set of applications, the properties of (strept)avidin proteins have been altered using protein engineering. Structural modifications (Chu et al., 1998; Hytönen et al., 2005b; Nordlund et al., 2004, 2005a), point mutations (Aslan et al., 2005; Avrantinis et al., 2002; Chilkoti et al., 1995a, 1995b; Dixon and Kollman, 1999; Freitag et al., 1999; Laitinen et al., 2006, 2007; LeTrong et al., 2003; Pazy et al., 2001; Riihimäki et al., 2011a), thermostability modifications (González et al., 1999; Hytönen et al., 2005a), increased proteolytic stability (Ellison et al., 1995), stability optimization over a wide range of pH (Green, 1975) and the immobilization of proteins using streptavidin-derived peptide tags (Hu et al., 2009) have all been successfully demonstrated for (strept)avidin without destroying the β-barrel structure or their high affinity to Btn.

The advantages of the strept(avidin)–Btn technology have obvious applications in bio(nano)technology. For example, for protein immobilization using the surface plasmon resonance technique, (strept)avidin has been adsorbed to a surface and used to attach biotinylated proteins (Hutsell et al., 2010); biotinylated nucleotides, acting as a scaffold for (strept)avidin and its conjugates, have been used to construct DNA-based nanomaterials (Li et al., 2004; Niemeyer et al., 1999; Park et al., 2005; Yan et al., 2003); gene detection via biotinylated DNA probes binding to chicken Avd attached to a sensor surface (Jin et al., 2009); and manipulation of the binding properties of (strept)avidin-Btn have been used to construct oriented biotinylated streptavidin-lipid layers (Blankenburg et al., 1989; Darst et al., 1991). Streptavidin-Btn complexes have also been used to create a molecular valve by attaching Btn to a nanotube (Hinds et al., 2004). Functioning nanowires have been possible with the help of streptavidin, which can be used to sense biotinylated DNA by measuring the resistance of the wire (Ramanathan et al., 2005). Avd attached to poly(ethyleneimine)-labelled 2-iminobiotin was used to generate layer-by-layer assembly (Inoue et al., 2005). Another study by Caswell *et al.* (Caswell et al., 2003) and Dimitrijevic *et al.* (Dimitrijevic et al., 2005) showed that (strept)avidin-Btn can be used as a self-assembling connector between nanostructures. Dual-chain (dc) Avds and single-chain (sc)Avds (see section 2.7.2) recognizing multiple ligand types in addition

to Btn have been developed with the purpose of building materials by linking *e.g.* multiple ligand-linked proteins (Leppiniemi et al., 2011).

Due to the high alkaline pI of chicken Avd, and because it is also glycosylated in some systems, chicken Avd undergoes undesired interactions with negatively charged macromolecules. To avoid these issues, engineered forms of chicken Avd and neutral streptavidin have replaced chicken Avd in many applications, despite being a more expensive reagent in comparison to the native Avds (Bayer and Wilchek, 1994; Marttila et al., 1998, 2000; Wilchek and Bayer, 1990).

## 2.7 Engineered avidins

Due to their stable nature, small size and high affinity for Btn, Avds are highly amenable to genetic engineering. For example, both increased stability and reduced immunogenicity have been achieved (Määttä, 2010). The Btn-binding site in Avds is often targeted, and mutants have been created with altered binding affinities for Btn and for recognition of novel ligands, *e.g.* steroids (Riihimäki et al., 2011a), similar to anticalins, artificial lipocalins that were also engineered to bind novel ligands (Skerra, 2008). Avds have also been engineered to create Avd mutants with varied pIs (Marttila et al., 1998), changing antigenic properties, eliminating glycosylation sites (Hytönen et al., 2004; Marttila et al., 1998, 2000), lowering/eliminating affinity to Btn (Laitinen et al., 1999; Morag et al., 1996; Reznik et al., 1998; Takakura et al., 2013), deactivating the binding site (Howarth et al., 2006; Määttä et al., 2008), altering the ligand specificity (Leppiniemi et al., 2011; Riihimäki et al., 2011a) and stability (weakened stability, increased protease sensitivity), lowering/increasing the melting temperature, and modifying the quaternary structure and topology (Howarth et al., 2006; Laitinen et al., 2003; Lim et al., 2013; Wu and Wong, 2005); discussed in (Laitinen et al., 2006).

**2.7.1 Chimeric avidins** – Chimeric Avds were designed by picking a part of one Avd and merging it with another Avd, with properties from both of the parent Avds. For example, chimeric streptavidin tetramers combining wt streptavidin with genetically engineered streptavidin (N49C, H127C or W120A mutant) subunits were constructed by Chilkoti *et al.* in 1995

(Chilkoti et al., 1995b) to be utilized as tools for bioseparation and drug delivery. Hytönen *et al.* created a chimeric Avd (ChiAVD), where the chicken Avd β4 strand and its adjoining loops were replaced with the corresponding section from AVR4, to combine the high affinity Btn-binding properties of Avd with higher thermostability of AVR4 (Hytönen et al., 2005a). Since then, several chimeric Avds have been created and studied by Prof. Emeritus Kulomaa's and Assoc. Prof. Hytönen's lab (Määttä et al., 2011; Riihimäki et al., 2011b; Taskinen et al., 2014).

**2.7.2 Circularly permuted avidins (cpAvds)** – The influence of the loops to the ligand affinity of (strept)avidin has been carefully studied (Pazy et al., 2002), and a decrease in Btn-binding affinity of streptavidin by L3,4 loop deletion was already shown in an earlier study by (Chu et al., 1998; Ellison et al., 1995). These flexible loops regulate the ligand/solvent traffic inside the binding site with 'open' and 'close' loop conformations: the open loop conformation leads to ligand binding, and the closed loop conformation prevents solvent access, and minimizes ligand dissociation. For example, in streptavidin, the shorter L3,4 loop, the longer L5,6 loop, and the absence of a few H-bonds due to the difference in Btn-binding residues, have been shown as the major reasons for streptavidin's weaker Btn-binding affinity in comparison to chicken Avd. The HABA-binding affinity is also likely to be affected by the same structural differences within (strept)avidin (Green, 1990; Repo et al., 2006).

Two circularly permuted forms of chicken Avd (cpAvd5→4 and cpAvd6→5) have been designed by Nordlund and co-workers, where the original N- and C-termini were connected to each other via a linker and the L4,5 loop for cpAvd5→4 and L5,6 loop for cpAvd6→5 were cleaved to create the new termini (Nordlund et al., 2004) (see below). Similarly, Määttä and co-workers, created cpAvd4→3 by cleaving the L3,4 loop of chicken Avd, and the original N- and C- termini were joined via a linker. The cpAvd4→3 protein had significantly reduced Btn-binding affinity ($K_d$~1.4±0.3×$10^{-8}$ M) and a 1.5 fold increase in its HABA-binding affinity ($K_d$~5.4±0.7×$10^{-6}$ M) (Määttä et al., 2008).

**2.7.2.1 Dual chain avidins (dcAvds)** – Nordlund and coworkers (Nordlund et al., 2004) created cpAvds (cpAvd5→4 and cpAvd6→5) with

two distinct and independently modifiable ligand-binding sites held together in one polypeptide chain. The original N- and C- termini of the Avd, in both the cpAvds, were joined via a six residue linker (GGSGGS), while the new C-terminus of cpAvd5→4 was joined with the N-terminus of cpAvd6→5 via a three residue linker (SGG) to join the two different cpAvds in a single polypeptide chain to create a dual chain Avd (dcAvd). The N- and C- termini of the dcAvd were positioned close to each other in the 3D structure. This technique makes it possible to use two independent Avd subunits with variable binding affinities for different ligands, and connect them via a single polypeptide chain to create a dcAvd with two distinct binding sites. The dcAvd proteins have a β-barrel structure and dimerize, forming a structure similar to tetrameric Avds although slightly less stable. When the binding sites correspond to that in chicken Avd, the Btn-binding affinities are similar to that for chicken Avd (Figure 6).

**2.7.2.2 Single chain avidins (scAvds) –** Nordlund and coworkers in 2005 (Nordlund et al., 2005a) also created circular permutants in which two dcAvds were joined together by a twelve-residue peptide linker forming a single chain Avd (scAvd) (Figure 6). Thus, four independently modifiable binding sites were joined together in a single polypeptide chain and the scAvds had similar binding affinities and thermal stability to the dcAvds (Nordlund et al., 2005a). The approach was used to combine the binding sites from various Avds into the same polypeptide chain, constructing a protein binding two or more ligands or even with different affinities for the same ligand (Nordlund et al., 2005a). A similar approach has also been applied to modifications of streptavidin (Aslan et al., 2005).

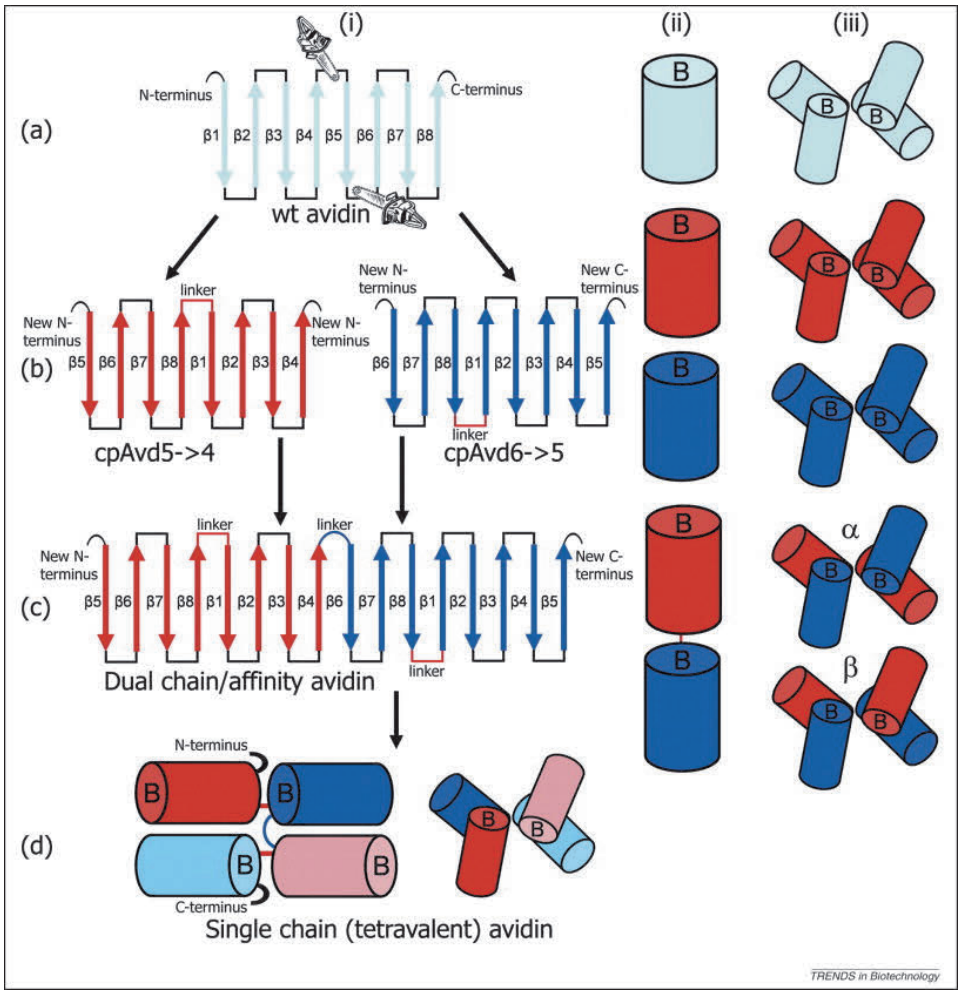


***Figure 6: Engineering strategy of dual chain (dc) and single chain (sc) Avds from (Laitinen et al., 2007).*** *(a) Chicken Avd topology (i), its tertiary (ii) and quaternary structure (iii). (b) Two circularly permuted mutants were generated by: one with the new termini between L4,5 loop region (upper chain saw, cpAvd5→4); and the other between L5,6 loop region (lower saw, cpAvd6→5). Both cpAvds had four identical subunits. (c) The C-terminus of cpAvd5→4 was joined to the N-terminus of cpAvd6→5 to generate a dcAvd. These dcAvds can combine to form two different quaternary structures or can be modified to have subunits from different Avds. (d) Two dcAvds were fused together to generate a scAvd, which consists four independently modifiable domains usable to combine other Avds with different ligand-binding properties. Reprinted with permission from Elsevier.*

**2.7.3 Steroid-binding avidins (sbAvds)** – Riihimäki and coworkers in 2011 (Riihimäki et al., 2011a) started development of chicken Avd-based mutants, capable of binding steroids. The steroid-binding sbAvd-1 and sbAvd-2 were chosen from phage display (Smith, 1985) selection and panning against testosterone. For sbAvd-1, Riihimäki and coworkers (Riihimäki et al., 2011a) constructed a phage display library, Avd L1,2, where the residues of the L1,2 loop region of chicken Avd were allowed to be randomly replaced by other amino acids. The sbAvd-1 carried the mutations D13R, L14M, G15N, and S16H in the L1,2 loop. To further reduce the Btn-binding affinity of sbAvd-1, another library, sbAvd-1 L3,4, was constructed where the residues of L3,4 loop in sbAvd-1 were allowed to be randomly replaced with other amino acids except tryptophan, glutamine, glutamate, methionine and lysine. The sbAvd-1 variant, sbAvd-2, carried additional mutations T35A, A36T, and T38N in the L3,4 loop regions along with the L1,2 loop region mutations from sbAvd-1; sbAvd-2 showed clear binding to testosterone and progesterone along with a reduced binding affinity to Btn in a microplate assay (Riihimäki et al., 2011a). In addition, unlike sbAvd-1, free Btn did not affect the binding of testosterone and progesterone to sbAvd-2 (Riihimäki et al., 2011a).

# 3. Aims of the study

The aim of this study was to determine the X-ray structures of natural and engineered Avds that recognize ligands other than Btn in order to elucidate the structural and functional features responsible for ligand recognition. These ligands included peptide ligands, steroids and other small molecules. These novel Avds will be helpful in developing and designing novel protein scaffolds for modern bio(nano)technological applications.

The specific aims of this study are as follows:

I. To characterize the molecular structure of novel, artificial, chicken Avd -based receptors, termed "antidins" that bind non-Btn ligands, such as steroids (publication I, manuscript II).

II. To analyze the binding of selected antidins with novel ligands *e.g.* progesterone, through computational molecular docking (manuscript II).

III. To characterize the molecular structure of core-bradavidin in complex with Btn, and produce computational models of other native, non-chicken Avd based Avds, *e.g.* rhodavidin and bradavidin A2, which also have the Brad-tag sequence at their C-terminus (publication III).

# 4. Materials and methods

The materials and methods, summarized below, are described in detail in publication I, III and manuscript II.

## 4.1 Crystallization and data collection (I, II, III)

Crystals (I, II, III) were obtained using the sitting drop vapor diffusion method (McPherson, 1982). Drops with varying proportions of protein solution and screening solutions were prepared with the mosquito® liquid handling robot (TTP Labtech) for the apo-form (I) and the progesterone bound sbAvd-2 (I117Y) form (II), and manually for the core-bradavidin–Btn complex (III). The crystals used for data collection typically appeared within a few weeks of incubation at 21°C in the temperature-controlled incubator (RUMED® model 3201). The crystals were initially analyzed using a PX Scanner (Agilent Technologies) (I, II) to check for diffraction and salt crystal detection. For data collection, cryoprotectant (CryoProtX™, Molecular Dimensions) for (I, II) and 100% glycerol for (III) was added to the crystallization drop just prior to fishing them out from the crystallization drop and freezing in liquid nitrogen. Data were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France (I, II) and MAX-LAB, Lund, Sweden (III).

## 4.2 Structure determination and refinement (I, II, III)

Data were processed using XDS (Kabsch, 1993). Molecular replacement was made using Phaser (McCoy et al., 2007) within CCP4i (Potterton et al., 2003; Winn et al., 2011), producing the initial phase estimates for the structure factors (I, II, III). The space group of the apo-form as well as ligand-bound sbAvd-2 (I117Y) mutant structures were confirmed to be $P2_12_12$ (I, II), and that of the core-bradavidin–Btn complex was confirmed to be $P2_12_12_1$ (III). The initial refinement of the structures was done with Refmac5 (Murshudov et al., 2011) and then manual editing used Coot (Emsley and Cowtan, 2004), including addition of water and ligand molecules. Solvent atoms were added using ARP/wARP (Lamzin and Wilson, 1993; Langer et al., 2008; Morris et al., 2003; Perrakis et al., 1999)

and later manually in Coot (Emsley and Cowtan, 2004) (I, II, III). A few refinement cycles for core-bradavidin–Btn complex were also done in Phenix (Adams et al., 2002, 2010; Zwart et al., 2008) (III). Coot (Emsley and Cowtan, 2004) and MolProbity (Davis et al., 2007) were used to validate the final structure (I, II, III). PyMOL (Schrödinger, 2010) and Bodil (Lehtonen et al., 2004) were used for visualization. Final structure coordinates and structure factors were deposited into Protein Data Bank (Berman et al., 2000, 2002) with PDB codes 4U46 (sbAvd-2 (I117Y), at 1.95 Å resolution) (I), 5LUR (sbAvd-2 (I117Y) in complex with progesterone, at 2.8 Å resolution) (II) and 4BBO (core-bradavidin in complex with Btn, at 1.60 Å resolution) (III).

## 4.3 Homology modelling (II, III)

All protein sequences were retrieved from the NCBI sequence database (http://ncbi.nlm.nih.gov/). The crystal structure coordinates used as templates – the sbAvd-2 (I117Y)–progesterone complex structure [PDB:5LUR] and the Avd-Btn complex structure [PDB: 1AVD] for two different sets of models for sbAvd-7–9 (II) and the wt bradavidin structure [PDB:2Y32] for rhodavidin and bradavidin A2 (III) – were obtained from the PDB (Berman et al., 2000, 2002). Structure-based sequence alignments were made using Malign (Johnson and Overington, 1993) in Bodil (Lehtonen et al., 2004) and later formatted for use by Modeller 9.14 (Sali and Blundell, 1993) to perform the model building. Ten tetrameric models were created for each protein that were internally ranked by Modeller using Molecular PDF (molpdf) score and Discreet Optimized Protein Energy (DOPE) scoring system. MolProbity (Davis et al., 2007) was also used to check geometrical and structural parameters. The Ramachandran plot was observed for each model and more than 96% of all the residues were in the favored regions of the plot. Structure visualization was made via PyMol (Schrödinger, 2010). Final structure selection was done based on visual analysis and the results of the Ramachandran plot.

## 4.4 Molecular docking (II)

Ligands were docked in Maestro 11.3 of the Schrödinger suite (Schrödinger Release 2017-3: Maestro, Schrödinger, LLC, New York, NY, 2017). Ligands were imported from the NCBI PubChem database (https://pubchem.ncbi.nlm.nih.gov/) along with the model structure (sbAvd-7–9) and the experimentally determined sbAvd-2 (I117Y) crystal structure (with a modeled L3,4 loop region and no progesterone). The protein preparation wizard application in Maestro 11.3 was used for protein structure optimization and energy minimization using the force field OPLS_2005. Ligands were minimized by the LigPrep application using the OPLS_2005 force field in Maestro 11.3. Protein-ligand docking was performed by the Induced Fit Docking (IFD) application (Farid et al., 2006; Sherman et al., 2006a, 2006b) in Maestro 11.3.

## 4.5 PCA and variance analysis (II)

The conformations of different poses of the docked ligands were compared to each other using principal component analysis (PCA). The distances of each poses from one another were input into a set of C programs (fixrmsd; PCA, MS Johnson). The distances for all pairs of poses were calculated as the RMSD of their coordinates. The PCA program output the points as a PDB-style coordinate file where each point corresponded to a pose. The program displayed each point such that the variance among the data is a maximum. The data were displayed within the most informative three dimensions determined by the three largest eigenvalues. The PDB file was visualized in Bodil (Lehtonen et al., 2004).

# 5. Results

The results summarized below present the author's contribution in the publications I-III from the closely collaborative project.

## 5.1 Structure of steroid-binding avidin (sbAvd)-2 (I117Y) (I, II)

### 5.1.1 Structure of apo sbAvd-2 (I117Y) (I)

We have determined the X-ray structure of a novel, steroid-binding Avd mutant, sbAvd-2 (I117Y), at 1.95 Å resolution (Table 4, publication I, for structure determination statistics) [PDB:4U46] in the ligand-free apo form (Figure 7). The crystal used for data collection had two monomers per asymmetric unit, but the protein assembly was tetrameric. The sbAvd-2 (I117Y) structure was designed based on chicken Avd and, despite containing major differences in the L1,2 and L3,4 loop regions as a result of selection from a phage display library (see below), the sbAvd-2 (I117Y) structure retained the β-barrel structure characteristic of all Avds.

Owing to the importance of the L1,2 and L3,4 loops in chicken Avd–Btn binding, these loop regions were selected by our collaborators (Prof. Emeritus Markku Kulomaa's and Assoc. Prof. Vesa Hytönen's research groups), for creating phage display gene libraries, where the loop residues were randomly replaced by other amino acids. The first library consisted of randomized residues over the L1,2 loop; the second library consisted of randomized residues for the L3,4 loop. Steroid binding Avds were identified from the libraries by phage display panning against a testosterone-coated surface. The chicken Avd variant, sbAvd-2, selected for lower Btn binding and higher progesterone binding, had the following mutations: D13R, L14M, G15N and S16H in the L1,2 loop region and T35A, A36T and T38N in the L3,4 loop region (Riihimäki et al., 2011a). Additionally, a site-directed mutation, I117Y, proposed on the basis of Tyr115 in AVR4 (Hytönen et al., 2004), was introduced into the β8 strand of sbAvd-2 to increase thermostability.

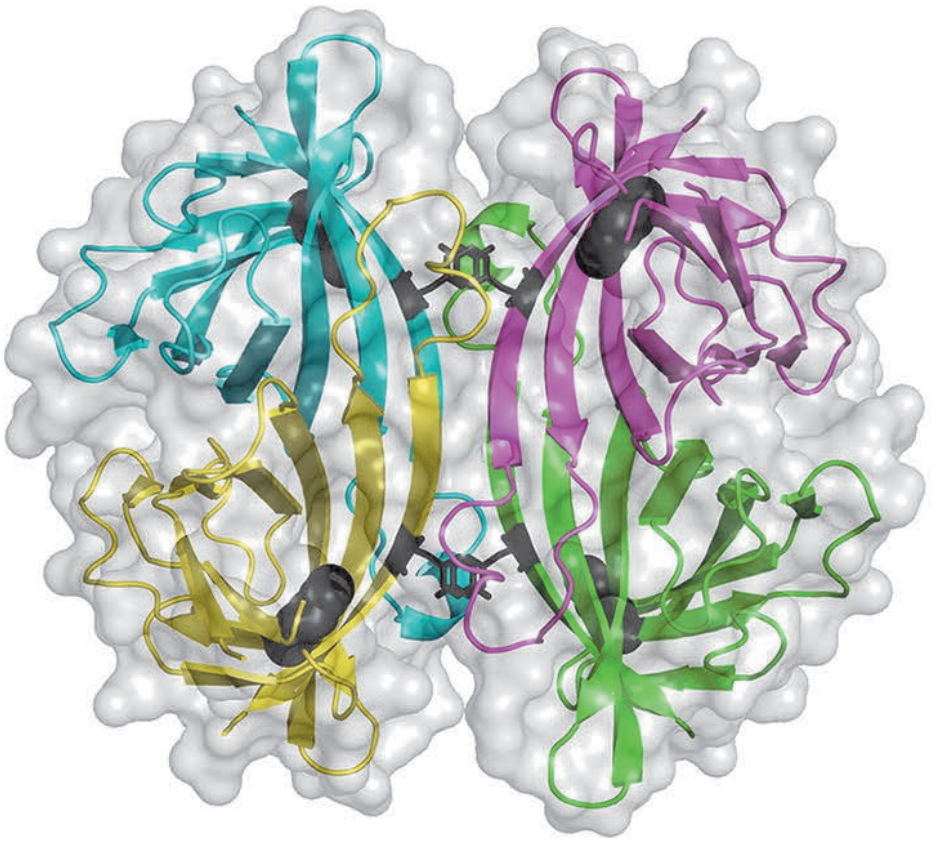

***Figure 7: X-ray structure of apo sbAvd-2 (I117Y) [PDB:4U46] (publication I).*** *The tetrameric structure of the sbAvd-2 (I117Y) is shown with transparent surface. The Tyr117 residues at the interface are shown as black sticks and the His16 residues inside the binding site are shown as black spheres. The subunits of the tetramer are colored cyan (subunit 1), green (subunit 2), magenta (subunit 3) and yellow (subunit 4). Reprinted with permission from American Chemical Society.*

Residues Asn38-Asn42 of the L3,4 loop in sbAvd-2 (I117Y) could not be built into the crystal structure due to non-interpretable electron density arising from high thermal motion of the flexible loop. Unlike the chicken Avd-Btn complex structure [PDB: 2AVI, 1AVD] (Livnah et al., 1993a; Pugliese et al., 1993), where the L3,4 loop adopts a 'closed' conformation, acting as a lid to seal the Btn inside the binding site, the L3,4 loop in sbAvd-2 (I117Y) adopts an 'open' conformation, which was attributed to the absence of a ligand in the ligand binding site (Figure 4B, publication I). In

comparison to Ile117 in sbAvd-2, the engineered Tyr117 mutation, present at the interface in sbAvd-2 (I117Y) is involved in π-π stacking with Tyr117 of the neighboring subunit. Tyr117 also forms two H-bonds: one with the side chain of Lys94 of the neighboring subunit and another with a nearby water molecule, thereby increasing the thermostability of the sbAvd-2 (I117Y) mutant by ~ 15°C in comparison to sbAvd-2.

In the sbAvd-2 (I117Y) apo form, two mutations – T35A in the L3,4 loop and S16H in the L1,2 loop – were hypothesized to be important for reduced Btn-binding affinity ($K_d$~$10^{-9}$ M). In chicken Avd, Thr35 is H-bonded to the N3′ nitrogen atom of the ureido ring and Ser16 is H-bonded to the oxygen atom of the ureido ring of the Btn ligand. During superimposition of the structure of chicken Avd [PDB:1AVD] with the structure of the sbAvd-2 (I117Y) apo form [PDB:4U46], it was observed that the H-bonds formed by Thr35 and Ser16 in chicken Avd would have been missing in sbAvd-2 (I117Y) due to the mutations T35A and S16H. Moreover, Ala35 created a more hydrophobic environment inside the binding site of the sbAvd-2 (I117Y) protein that would make it less suitable for the ureido ring of an incoming Btn, and the imidazole side chain of His16 protruded inside the binding site partially occupying a space equivalent to the ureido ring oxygen atom of Btn (Figure 4C, publication I).

### 5.1.2 Structure of sbAvd-2 (I117Y)–progesterone complex (II)

We also determined the X-ray structure of sbAvd-2 (I117Y) in complex with progesterone at 2.8 Å resolution (Table 1, manuscript II) [PDB:5LUR] using a co-crystallization method. The complex structure of the sbAvd-2 (I117Y) protein has, as expected based on the apo structure, the characteristic β-barrel structure of Avds, with a progesterone ligand bound to each monomer; there were two monomers in the asymmetric unit of the crystal, but the assembly was clearly tetrameric, similar to the apo structure (Figure 8).

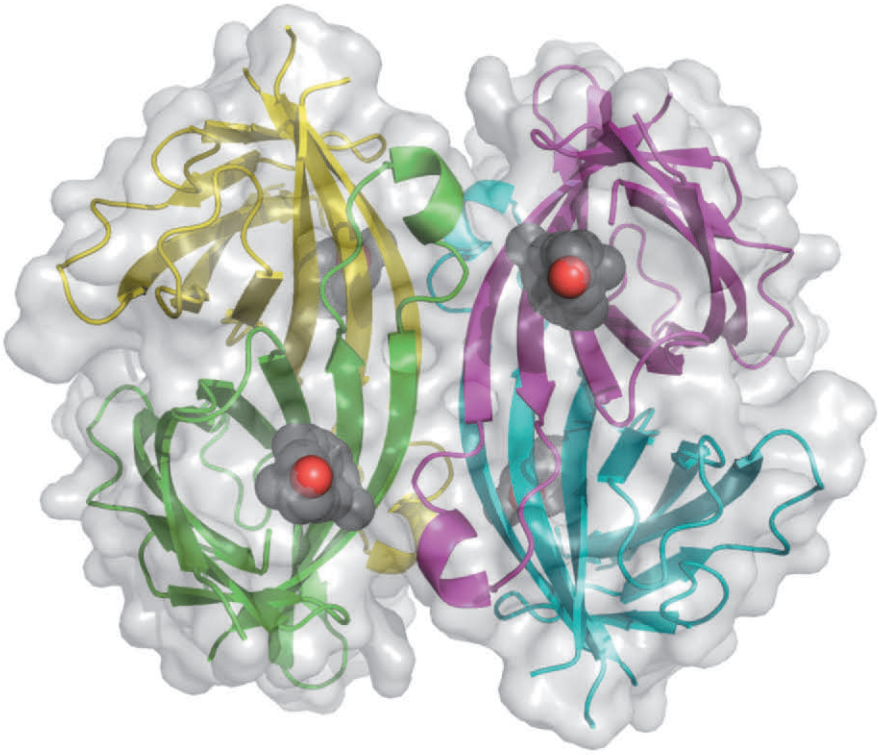

***Figure 8: X-ray structure of the sbAvd-2 (I117Y)-progesterone complex [PDB:5LUR].*** *The tetrameric structure of sbAvd-2 (I117Y) with bound progesterone is shown with transparent surface. The progesterone ligands inside the binding sites are shown as black spheres (carbon atoms). The four subunits are colored green (subunit 1), magenta (subunit 2), cyan (subunit 3) and yellow (subunit 4). The oxygen atoms of the progesterone ligands are colored red.*

The progesterone-binding mode of the sbAvd-2 (I117Y) crystal structure was different from our earlier hypothesis, which was based on automated docking of progesterone to sbAvd-2 (I117Y). In the docking analysis, the A-ring of the progesterone ligand (Figure 5a) faced the hydrophobic core of the binding site, and the L3,4 loop adopted a 'closed' conformation to seal and stabilize the ligand inside the binding site. However, in the crystal structure, the progesterone ligand enters the ligand-binding site 'D-ring first' – the oxygen atom of the 20-acetyl group of progesterone forms H-bonds with the Asn118 Nδ (2.4 Å) and Trp97 Nε (3.0 Å) atoms; the critical

His16 and Ala35 residues were involved in hydrophobic interactions with progesterone, along with Met14, Tyr33, Val37, Trp70, Phe72, Thr77, Phe79 and Trp110 (from the neighboring subunit) (Figure 3, manuscript II). In contrast with the results from automated ligand docking, the L3,4 loop of the sbAvd-2 (I117Y) complex structure clearly adopted an 'open' conformation, even though residues Asn38-Ser41 of this loop could not be built into the electron density due to high thermal motion.

The L1,2 loop in progesterone-bound sbAvd-2 (I117Y), crucial for ligand binding in all Avds, showed clear changes in conformation compared to the apo sbAvd-2 (I117Y) structure [PDB: 5LUR *vs.* 4U46]. In comparison with apo sbAvd-2 (I117Y), the His16 side chain of the progesterone-bound sbAvd-2 (I117Y) structure relocated to stabilize the incoming progesterone ligand and the conformation and position of the side chains of Arg13, Asn15 and Asn17 also altered, although these residues did not directly affect ligand binding. The Met14 side chain moved towards the binding site by 5.5 Å in order to bind the progesterone ligand (Figure 2, manuscript II). Unlike in the case of the apo sbAvd-2 (I117Y) [PDB:4U46], the electron density for the L1,2 loop region of sbAvd-2 (I117Y)–progesterone complex [PDB:5LUR] was clearly interpretable indicating that the progesterone ligand stabilized the conformation of the L1,2 loop. Unfortunately, the Tyr117 mutation, added to improve the thermostability of the protein and located at the IF1,3 interface, was found to lower the binding affinity of the sbAvd-2 (I117Y) antidin to progesterone in a fluorometric assay. The direct effect of this mutation for the structure was not clear; however, Tyr117 could indirectly affect the progesterone binding by altering the conformation/location of the next residue, Asn118, which forms a H-bond with the progesterone ligand in the sbAvd-2 (I117Y) complex structure.

## 5.2 Docking studies on selected antidins (II)

Our collaborators (Prof. Emeritus Markku Kulomaa's and Docent Vesa Hytönen's research groups), in collaboration with our laboratory (structural analysis), have developed a number of artificial, chicken Avd-based antidins. In comparison to chicken Avd, these antidins contained major differences in their loop regions near the ligand-binding site; a few antidins contained additional mutations of other residues, too (see Table 2, Publication I; Table 2, manuscript II for a list of all the mutated residues in the studied antidins). All of the studied antidins were tested for their binding affinity with progesterone and Btn using a fluorometry-based assay. Based on the results of this assay, we selected three high-affinity progesterone binders (Table 3) – sbAvd-7 ($K_d$~5 nM), sbAvd-8 ($K_d$~11 nM), sbAvd-9 ($K_d$~13nM) – which we modeled (details in section 5.4) and docked Btn and progesterone in order to gain insight on their possible ligand-binding modes at the atomic level.

After the structural characterization of the sbAvd-2 (I117Y)–progesterone complex structure, we expected that the sbAvds having the same mutations in the L1,2 loop region – Arg13, Met14, Asn15, His16 (RMNH) – should have a similar binding mode, progesterone entering the ligand-binding site 'D-ring first' and the L3,4 loop adopting an open conformation. In accordance with these assumptions, our docking analysis showed that four out of eleven (sbAvd-7), six out of eight (sbAvd-8) and seven out of sixteen (sbAvd-9) progesterone poses were docked in a similar orientation and depth as the progesterone was observed in the ligand-bound sbAvd-2 (I117Y) crystal structure (Figure 5, manuscript II).

*Table 3: List of mutations in the selected high-affinity progesterone binders, in comparison to chicken Avd structure [PDB:1AVD] and the sbAvd-2 (I117Y)–progesterone complex structure [PDB:5LUR].*

| Chicken Avd | Sbavd-2 (I117Y) | Sbavd-7 | Sbavd-8 | Sbavd-9 |
|---|---|---|---|---|
| **D13** | R | R | R | R |
| **L14** | M | M | M | M |
| **G15** | N | N | N | N |
| **S16** | H | H | H | H |
| **Y33** | - | - | F | - |
| **T35** | A | A | - | Y |
| **A36** | T | S | Y | Y |
| **V37** | V | L | S | L |
| **T38** | N | N | Y | P |
| **T77** | - | A | A | A |
| **I117** | Y | - | - | - |

Despite extensive visual analysis of the ligand poses in the docking complexes, we were not able to completely explain the difference in the binding affinities of sbAvd-7, sbAvd-8 and sbAvd-9, in comparison to sbAvd-2 (I117Y) and each other, at the atomic level. In each of the antidins sbAvd-7–9, varying interaction networks were observed for every progesterone pose in each docked complex. The potential hydrophobic/van der Waals interactions, were not trivial to predict despite the unique set of mutations in the L3,4 loop region in each of the antidins sbAvd-7–9. As a reference, we manually removed the ligand from the crystal structure of the sbAvd-2 (I117Y)–progesterone complex [PDB:5LUR] and docked it back into the ligand-binding site – even in that case, the docking poses produced were different to the ligand pose observed in the crystal structure, indicating the challenges of docking a pseudo-symmetrical steroid, such as progesterone in an Avd-like binding pocket and/or the limitations of the state-of-the-art docking programs.

In the case of Btn docking with sbAvd-7–9, we observed that the His16 residue inside the binding pocket clearly hindered Btn binding in these antidins. This was in agreement with our earlier prediction after the

analysis of the sbAvd-2 (I117Y) complex structure, that His16 in the L1,2 loop is one of the key residues to reduce Btn-binding. In addition to the role of His16, the mutations in the L3,4 loop further reduced the Btn-binding affinity of sbAvd-7–9. For example, in the case of sbAvd-9, a bulky tyrosine residue at position 35 extending directly inside the ligand-binding site restricted the entry of Btn.

## 5.3 Structure of core-bradavidin–biotin complex (III)

Bradavidin is a tetrameric protein isolated from the nitrogen-fixing bacterium *Bradyrhizobium diazoefficiens* (Delamuta et al., 2013; Nordlund et al., 2005b). Wild-type bradavidin is 138 amino acids in length, and its C-terminal residues $^{129}$GSEKLSNTKK$^{138}$, known as the 'Brad-tag', acts as an intrinsic peptide ligand that binds to the ligand-binding site of the adjacent subunit in the tetramer, as was recently detailed in our laboratory by determining the X-ray structure of wt bradavidin (Leppiniemi et al., 2012).

Engineered core-bradavidin lacks the C-terminal residues Gly114-Lys138, thus removing the Brad-tag (Gly129-Lys138). In publication III, we reported the X-ray crystal structure of the core-bradavidin–Btn complex at 1.60 Å resolution (Figure 9) [PDB:4BBO] (Table 1, publication III for structural determination statistics). Despite the low sequence similarity – 35% percent identity – between chicken Avd and core-bradavidin, most of the Btn-binding residues are conserved in core-bradavidin (Figure 10). The exceptions include Asp107 (Asn118 in chicken Avd) and Asn33 residues (Thr35 in chicken Avd).

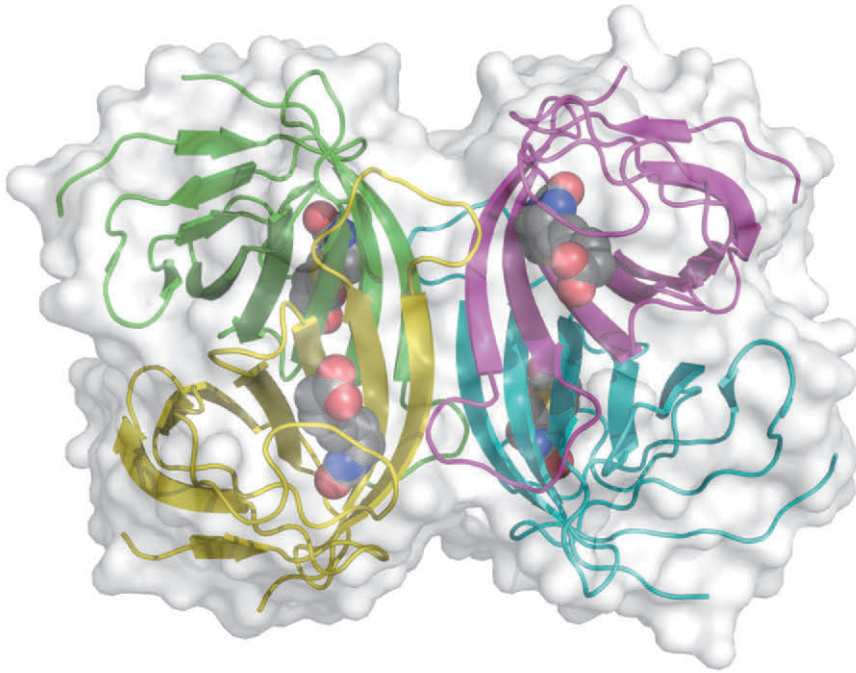

***Figure 9: X-ray structure of the core-bradavidin-Btn complex [PDB:4BBO].*** The tetrameric structure of core-bradvidin with bound progesterone is shown with transparent surface *The Btn ligand found in all the four binding sites of the tetrameric structure is shown as black spheres (carbon atoms). The four subunits are colored yellow (subunit 1), cyan (subunit 2), magenta (subunit 3) and green (subunit 4). The nitrogen atoms are colored blue, oxygen atoms red and sulfur atoms yellow.*

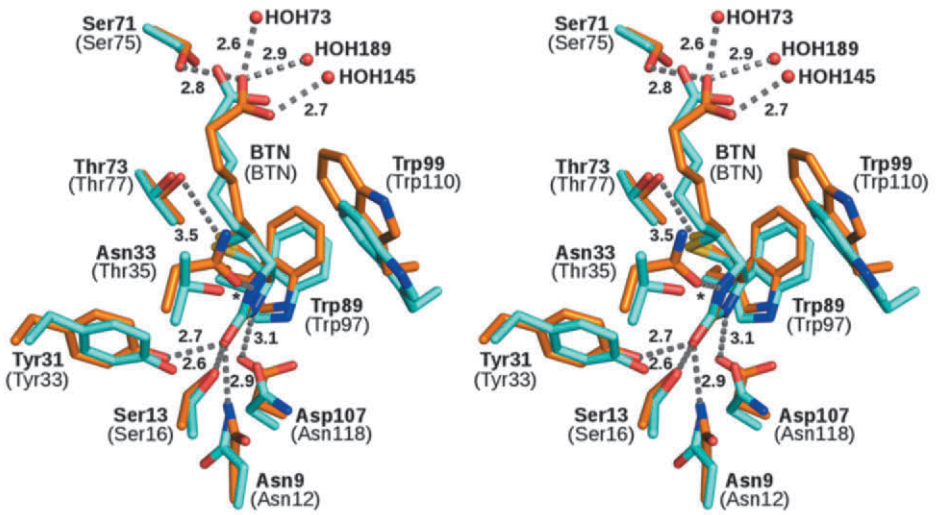


***Figure 10: Comparison of the Btn-binding residues of core-bradavidin [PDB:4BBO] (orange carbon atoms, bold labels) and chicken Avd [PDB:1AVD] (cyan, labels in brackets) presented as a stereo view (publication III).*** *The Cα traces of both the structures were superimposed. Trp99 (Trp110 in chicken Avd) is shown from the neighboring subunit, other residues are from subunit 1. The bound Btn ligands are shown as sticks. Nitrogen atoms are colored blue, oxygen atoms red and sulfur atoms yellow. Water molecules are shown as red spheres. H-bonds are drawn as grey dashed lines and distances are in Ångströms. (*=2.8 Å). Reprinted with permission from PLOS ONE.*

The major structural difference between the wt bradavidin structure and the core-bradavidin structure is in the L3,4 loop region. In wt bradavidin, the L3,4 loop adopts an 'open' conformation in order to accommodate the bulkier Brad-tag (Figure 11). In contrast, in the core-bradavidin–Btn complex structure, the L3,4 loop adopts a 'closed' conformation, sealing Btn within the binding site. Hence, residues of the L3,4 loop, including Ser38, Cys39, Asp40 and Glu41, undergo major structural/conformational changes in the core-bradavidin structure in comparison to wt bradavidin structure (Figure 7, publication III).

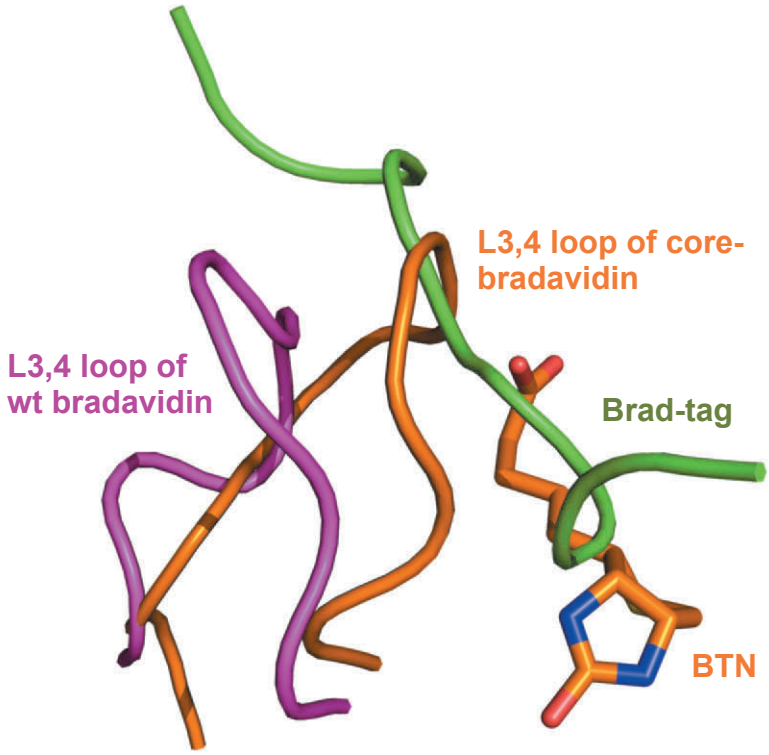


***Figure 11: Comparison of the L3,4 loop of core-bradavidin [PDB:4BBO] (subunit 1, orange) and wt bradavidin [PDB:2Y32] (subunit 1, magenta; subunit 3, green).*** *The Cα traces of both the structures were superimposed. Nitrogen atoms are colored blue, oxygen atoms red and sulfur atoms yellow.*

A disulphide bridge near the Btn binding site is a feature commonly observed in the dimeric Avds, for example, rhizavidin (Meir et al., 2009), hoefavidin (Avraham et al., 2015), shwanavidin (Meir et al., 2012) and bradavidin II (dimeric and tetrameric forms) (Leppiniemi et al., 2013). The disulphide bridge helps stabilize the ligand-binding site. Bradavidin is the first *tetrameric* Avd reported to have a disulphide bridge, Cys39 (L3,4 loop) – Cys69 (L5,6 loop), located near the binding site (Leppiniemi et al., 2012). In core-bradavidin, the Cys39 residue is not structurally equivalent to the cysteine residue observed in the dimeric Avds (Figure 12), whereas the other cysteine residue, Cys69, is structurally conserved even though the L5,6 loop conformation in rhizavidin, hoefavidin, shwanavidin, and bradavidin II is different. In wt bradavidin, the conformation of the side

chain of Cys39 changes to assist in the 'opening' of the L3,4 loop in order to accommodate the Brad-tag. The Asp40 residue also seems to play an important role in this regard – the side chain of Asp40 is flipped in wt bradavidin, in comparison to core-bradavidin (Figure 5, publication III). Thus, besides the possible role of the disulphide bridge in tetrameric bradavidin in stabilizing the binding site, it also appears to have a role to aid opening/closing of the L3,4 loop during ligand binding.

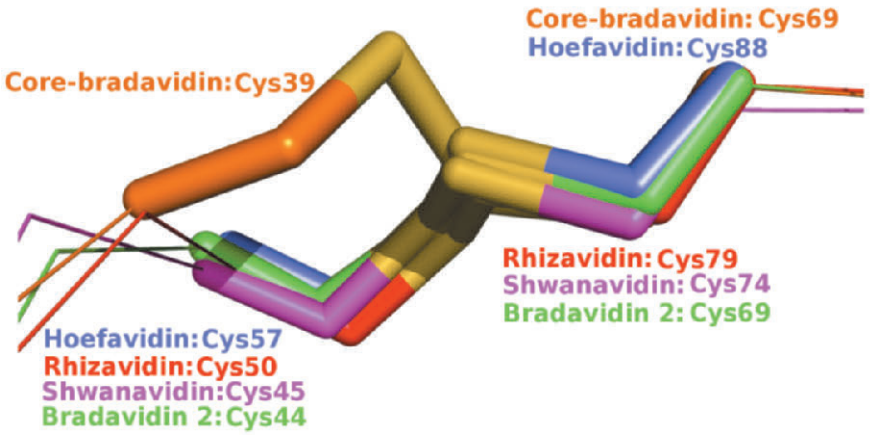


***Figure 12: Disulphide bridge in the ligand-binding site of core-bradavidin [PDB:4BBO] (orange carbon atoms), rhizavidin [PDB:3EW2] (red), shwanavidin [PDB:3SZJ] (magenta), bradavidin 2 [PDB:4GGZ] (green) and hoefavidin [PDB:4Z28] (blue).*** *The Cα traces of subunit 1 were superimposed. The sulfur atoms are colored yellow.*

## 5.4 Homology models of natural and engineered avidins (II, III)

The structures of several artificial antidins, based on chicken Avd, were modeled because we did not have their experimental structures; the models were used to visualize the consequences of the differences with chicken Avd and for the docking studies (II). We created two different set of models for each of the antidins sbAvd-7–9: one for docking progesterone, having an open L3,4 loop conformation, similar to the sbAvd-2 (I117Y)–progesterone complex structure [PDB:5LUR], and the other for docking

Btn, having a closed L3,4 loop conformation similar to the chicken Avd structure [PDB:1AVD].

In the case of the sbAvd-7–9 models for progesterone docking, the template used was the sbAvd-2 (I117Y)-progesterone complex structure [PDB:5LUR], in which the missing L3,4 loop residues were modeled in an open conformation. The sbAvd-2(I117Y)-progesterone complex structure [PDB:5LUR] provided details for the L1,2 loop region, which were similar in all three studied antidins, and for the L3,4 loop region, which was expected to be in an open conformation while binding progesterone.

For the sbAvd-7–9 models used for docking Btn, we used two templates: the chicken Avd structure [PDB:1AVD], and the L1,2 loop region from the sbAvd-2 (I117Y)–progesterone complex structure [PDB:5LUR]. Because Btn inside the Avd-binding site prefers to interact with the residues of the L3,4 loop in a 'closed' conformation, we used chicken Avd as the template, which has a closed L3,4 loop region while binding Btn. However, owing to the identical mutations – D13R, L14M, G15N and S16H – in the L1,2 loop region of sbAvd-7–9 and the sbAvd-2 (I117Y) structure [PDB:5LUR], we modeled the L1,2 loop region based on the ligand bound sbAvd-2 (I117Y) crystal structure (II).

In order to learn more about the mode of Brad-tag binding in other Avds containing the Brad-tag sequence, two structures were modeled using wt bradavidin as the template [PDB: 2Y32]: rhodavidin from *Rhodopseudomonas palustris* (Larimer et al., 2004) and bradavidin A2 from *Bradyrhizobium* sp. Ai1a-2 (III) (Figure S2, publication III). The rhodavidin and bradavidin A2 proteins are respectively 75% and 74% identical in sequence to bradavidin; both have an extended linker region between their β8-strand and the Brad-tag. Additionally, two residues in bradavidin A2 differ from bradavidin within the Brad-tag region: Ala131 (Gly129 in wt bradavidin) and Gly132 (Ser130 in wt bradavidin). In the modeled structures, these differences are unlikely to affect Brad-tag binding to rhodavidin and bradavidin A2 significantly, but they have not been tested experimentally.

# 6. Discussion

Streptavidin and chicken Avd, collectively known as strept(avidin), bind to Btn with one of the highest observed affinity for a ligand via non-covalent interactions; indeed, the dissociation constant for chicken Avd is $K_d \sim 10^{-15}$ M (Green, 1975). Strept(avidin) is also extremely stable against heat, pH and other denaturants (González et al., 1999; Green, 1975; Hytönen et al., 2005a). These properties form the basis of the strept(avidin)-Btn technology, which is utilized in a plethora of life science applications to purify, label, separate and target proteins, peptides and other molecules. In order to extend the range of applications utilizing this technology, strept(avidin) has been modified both chemically as well as genetically (Laitinen et al., 2006, 2007).

Even though the four (or two in dimeric Avds) binding sites of the Avds have a highly complementary fit to the Btn ligand, some Avds also bind non-Btn ligands, such as Btn analogs, azocompounds, and peptide ligands, all with reduced affinity in comparison to Btn. In this thesis, we focus on natural Avds binding intrinsic peptide ligands as well as artificial Avds, 'antidins', having tailored binding sites for steroids. Because dc and sc Avds have been engineered, binding observed towards non-Btn ligands could be incorporated as separate binding sites on two polypeptide chains or on a single polypeptide, yielding an Avd capable of simultaneously binding up to two (dc) or four (sc) different ligands, respectively. Such Avds could be utilized in various applications in bio(nano)technology where simultaneous recognition of multiple ligands is beneficial, for example in diagnostic applications.

## 6.1 Natural avidins binding peptide ligands

The binding of an intrinsic peptide ligand naturally in the Btn-binding site of Avds is an uncommon mechanism, currently observed in only a few prokaryotic Avds – tetrameric Avds: streptavidin (*Streptomyces avidinii*) (LeTrong et al., 2006) and bradavidin (*Bradyrhizobium diazoefficiens)* (Delamuta et al., 2013; Nordlund et al., 2005b); and dimeric Avds:

rhizavidin (*Rhizobium etli*) (Meir et al., 2009) and hoefavidin (*Hoeflea phototrophica*) (Avraham et al., 2015).

For example, in wt bradavidin, the C-terminal Brad-tag (Gly129-Lys138) acts as an intrinsic peptide ligand by attaching itself to the Btn-binding site of the neighboring subunit and *vice versa* [PDB:2Y32] (Leppiniemi et al., 2012). Core-bradavidin was engineered to remove the C-terminal residues Gly114-Lys138, which includes the Brad-tag sequence, from wt bradavidin. In publication III, we reported the structure of core-bradavidin in complex with Btn [PDB:4BBO] at 1.60 Å resolution. With the characterization of the core-bradavidin–Btn complex [PDB:4BBO], and the previously determined wt bradavidin–Brad-tag complex [PDB:2Y32], we now have two bradavidin crystal structures detailing the binding mode of two very different ligands of this Avd.

The two bradavidin crystal structures [PDB: 4BBO and 2Y32] were visually characterized on a graphical 3D workstation and using computational tools to identify major differences undertaken by bradavidin in order to bind both the Brad-tag and Btn. In core-bradavidin, the L3,4 loop adopted a 'closed' loop conformation to bind Btn, whereas, in wt bradavidin, the L3,4 loop adopted an 'open' loop conformation to bind the bulkier Brad-tag sequence. A clear conformational change in the Cys39 side chain is observed between wt bradavidin and core-bradavidin, suggesting a possible role for the disulphide bridge (Cys39-Cys69) in enabling differential ligand binding. To test this possibility, we mutated Cys39 and Cys69 to alanine residues (core-bradavidin CC mutant). However, we did not observe any significant differences on Btn-binding between core-bradavidin and the CC mutant. Although Brad-tag binding could not be tested experimentally, the low yield of the CC mutant hints at a possible role of the disulphide bridge in protein folding. Another core-bradavidin variant, core-bradavidin V1, was designed to mask the hydrophobic residues Leu51, Leu79 and Phe111 that were exposed to solvent in core-bradavidin because of its truncated C-terminus (missing the residues Ala128-Lys138 of wt bradavidin) and thought to affect solubility. Again, ligand binding was not significantly different from core-bradavidin, and solubility was not increased either. Ligand binding and characterization of both the CC mutant and the V1 variant were made in Docent Hytönen's lab (University of Tampere).

In order to understand the Brad-tag binding mode further, we modeled two Brad-tag containing Avds: rhodavidin from *Rhodopseudomonas palustris* (Larimer et al., 2004) and bradavidin A2 from *Bradyrhizobium* sp. Ai1a-2. Based on our modeling results, the Brad-tag binding mode of rhodavidin and bradavidin A2 were similar to wt bradavidin. The few amino acid differences at the beginning of the Brad-tag region and within the extended linker region in rhodavidin and bradavidin A2, in comparison to wt bradavidin, are likely not to affect the Brad-tag binding of rhodavidin and bradavidin A2, but this has not yet been tested experimentally.

Btn easily displaces peptide ligands during competitive binding assays. However, the presence of a peptide ligand hinders the Btn-binding affinity in Avds. The reason for the existence and the exact role of intrinsic peptide ligands in Avds have been discussed recently (Avraham et al., 2015; Leppiniemi et al., 2012) but the function of the intrinsic peptides is not entirely known. One possible reason for the presence of peptide ligands in bacterial Avds may be to help regulate Btn intake and release, suggesting a Btn-transporter role of Avd in the host organism, similar to BBPs (Hytönen et al., 2007). Avds may help distribute Btn inside the bacterial cell close to the sites where Btn is needed; for example, Btn molecules are cofactors for carboxylase enzymes, which are essential for gluconeogenesis and fatty acid synthesis (Dakshinamurti et al., 1985) and hence access to readily available Btn close to the site where these reactions take place could be necessary. Especially, the relatively low affinity Btn-binding bacterial Avds (*i.e.* not streptavidin) could have this biological role.

The X-ray structure of the core-bradavidin–Btn complex described in publication III did not explain how Btn replaces Brad-tag in the ligand-binding site of the wt bradavidin structure. To address this question, we also tried to co-crystallize wt bradavidin with Btn, however, no crystals were obtained, possibly due to the highly flexible Brad-tag region still present in solution, after being displaced by Btn. By rational mutagenesis of the Brad-tag, we sought to increase the binding affinity of the Brad-tag for core-bradavidin, but none of the Brad-tag mutants bound core-bradavidin tighter than the original Brad-tag according to differential scanning calorimetry analyses. The affinity of the Brad-tag for bradavidin could also be increased by rational mutagenesis of bradavidin itself, but this

has not been attempted so far. Similarly, directed evolution approaches may lead to a higher-affinity complex between the core-bradavidin–Brad-tag ligand pair that would be better suited for biotechnological assays, such as protocols where Brad-tag could be used as an alternative tag for the existing tags for purification of recombinant proteins, or for electrochemical biosensing, where the core-bradavidin–Brad-tag ligand pair can be used to build multilayered materials in bio(nano)technology.

## 6.2 Artificial avidin mutants binding steroid ligands

The high thermal stability ($T_m$~80°C), small size (~60 kDa) and low cost of production, as well as the architecture of the ligand-binding site, make chicken Avd an attractive, alternative receptor scaffold for the wide-range of applications where traditional antibodies are currently commonly used.

Receptor scaffolds can be classified into two broad structural classes: domain-sized scaffolds *e.g.* anticalins; and constrained peptide scaffolds *e.g.* avimers. Even though a number of protein scaffolds are being developed, the need for better protein scaffolds is on the rise. According to Vazquez-Lombardi and coworkers (Vazquez-Lombardi et al., 2015), the next-generation protein scaffolds face three major hurdles:

i) Most antibodies have a half-life of several weeks inside the body. In comparison, scaffolds are mostly passed out of the body after reaching the kidney.

ii) Even though the tissue penetration of scaffolds is much faster than antibodies due to their relatively small size, the dose toxicity and the manufacturing cost are higher. Currently, antibodies can easily access most tissues of the body with lower risk.

iii) Advances in antibody technology due to protein engineering have helped produce smaller antibodies and with longer half-lives, thus reducing the advantage of protein scaffolds to a certain degree.

However, protein scaffolds already hold a lot of benefits (Vazquez-Lombardi et al., 2015): owing to their smaller size, scaffolds can be locally

administered, *e.g.* in ocular diseases; innovative drug delivery devices can exploit their higher solubility; they can have multiple specificity for various ligands/drugs, *e.g.* anticalins and antidins; toxin-conjugated protein scaffolds can penetrate tumor tissues easily and upon delivery excreted out of the body much faster than antibodies.

Steroid-binding Avds (sbAvds) were recently developed in our collaborators' laboratory (Prof. Emeritus Markku Kulomaa's and Docent Vesa Hytönen's research groups, University of Tampere; Riihimäki et al., 2011a) and these chicken Avd-based receptors were named antidins (*anti*bodies + avi*dins*). In this study, 3D structure-based analysis of antidins was carried out to help further engineer and develop the steroid-binding antidins.

The first antidins, sbAvd-1 and sbAvd-2 were selected using phage display and panning against testosterone (Riihimäki et al., 2011a). For sbAvd-1, Riihimäki and co-workers constructed a phage display library (Avd L1,2 library), in which the residues Asn12-Ser16 of the L1,2 loop of chicken Avd were randomly replaced by other amino acids. The sbAvd-1 antidin carried the mutations D13R, L14M, G15N, and S16H and had a micromolar affinity to testosterone and progesterone but retained nanomolar affinity to Btn. To further reduce the Btn-binding affinity of sbAvd-1, the sbAvd-1 L3,4 library was constructed where the residues of the L3,4 loop were allowed to be randomly mutated except for tryptophan, glutamine, glutamate, methionine, and lysine; sbAvd-2 was selected from this library and carried the mutations T35A, A36T, and T38N in the L3,4 loop region in addition to the L1,2 loop region mutations of sbAvd-1 (Riihimäki et al., 2011a). In comparison to sbAvd-1, sbAvd-2 had increased affinity to testosterone (3  M) and progesterone (0.1  M) along with a reduced binding affinity to Btn (7  M) in a fluorometric assay.

In order to understand the binding mode of sbAvd-2, and of sbAvds both in general and in atomic detail, sbAvd-2 carrying the point mutation I117Y was crystallized in the apo form, and sbAvd-2 (I117Y) was co-crystallized with progesterone and testosterone. Crystals were obtained within a few weeks for sbAvd-2 (I117Y) without a ligand and after co-crystallization with testosterone and progesterone. However, crystals of size large enough

for mounting and of high enough quality for detecting initial diffraction using a PX Scanner, and for data collection at ESRF, were obtained only for the unligated form of sbAvd-2 (I117Y) [PDB:4U46] (I) and for sbAvd-2 (I117Y) co-crystallized with progesterone [PDB:5LUR] (II); the reason for not getting crystals with testosterone maybe related to the low solubility and hydrophobic properties of testosterone, which may have affected proper complex formation or crystal lattice formation.

The X-ray structure of the sbAvd-2 (I117Y)–progesterone complex [PDB:5LUR] helps us understand the mode of, and pinpoint the important residues involved in, progesterone binding. The L3,4 loop in the progesterone-bound complex structure [PDB:5LUR] is in an open conformation and residues Asn38-Ser41 lacked electron density, similar to the sbAvd-2 (I117Y) apo structure [PDB:4U46]. The open conformation of the L3,4 loop is not observed in Avd-Btn complex structures but is clearly needed to allow bulkier-than-Btn ligands to bind and fit to the otherwise too narrow binding pocket; in Btn binding the closed conformation of the L3,4 loop seals the hydrophobic moieties of Btn from the solvent and enables the perfect fit between the ligand and the "receptor" (extremely high affinity) but, in the case of progesterone, the size and shape of the ligand restrict the fit (lower affinity).

The X-ray structure of these two sbAvd structures allowed us to study the progesterone-binding mode at the atomic level and assisted in the further development of antidins through prediction of future mutations to improve the progesterone-binding affinity. For example, after detailed analysis of the sbAvd-2 (I117Y)–progesterone complex, we suggested mutations to key residues (*e.g.* A35V, V37L) near the binding site which may further contribute to progesterone binding by increasing the H-bonding network with the 3-keto group of progesterone (Figure 4, manuscript II).

**6.2.1 Docking analysis of ligands** – Out of the tested antidins in publication I and manuscript II (Table 2, publication I; Table 2, manuscript II), the antidins sbAvd-7–9 selected from AvLib-4 based on the progesterone panning bound progesterone with nanomolar affinity in a fluorometry-based assay. Thus, these antidins were selected for docking

analysis with progesterone and Btn in order to gain insight on their possible ligand-binding modes. Here, the aim was to identify the residues and interactions responsible for high-affinity progesterone binding and the residues that might lower the Btn-binding affinity. Even though our docking analysis could not explain the differences in the progesterone binding affinities, our Btn-docking analysis did support the role of His16 in hindering Btn binding in agreement with our earlier predictions based on the crystal structure of sbAvd-2 (I117Y) (I).

We assumed that all of the antidins derived from AvLib-3 and AvLib-4, *e.g.* sbAvd-1, sbAvd-2, and sbAvd-7–9, could bind progesterone as was seen in the progesterone-bound crystal structure of sbAvd-2 (I117Y) [PDB:5LUR]. However, progesterone has a "pseudo-symmetrical" structure, having a tetracyclic sterane skeleton with a methyl group present at C10 and C13, and polar functional groups attached at C3 and C17 (Moss, 1989; Steroids, 2017). This makes it difficult for docking programs to predict the orientation of steroids within the ligand-binding site of the antidins; and model structures with inherent errors used for docking do not make this task easier. Moreover, the rigidity of the sterane tetracyclic rings restricts the flexibility of the progesterone ligand, except at the functional groups, increasing the challenge even further. In many of the progesterone docking complexes of sbAvd-7–9, the progesterone ligand was 'A-ring' first inside the binding site, instead of 'D-ring' first (X-ray structure), as it was energetically favorable according to the docking algorithm (Figure 5a). A similar phenomenon was observed in our earlier automated docking analysis, in which the docking poses of progesterone were also inverted in comparison to the X-ray structure.

Similar challenges were also observed by Panek and coworkers (Panek et al., 2017), who tested the binding mode of progesterone and other steroids to human mineralocorticoid receptor and a bacterial monooxygenase through computational docking and molecular dynamic studies. In both cases, the steroids docked in at least two orientations, A-ring first and D-ring first, even though the X-ray structure used for their analysis had progesterone placed A-ring first.

Therefore, without experimental evidence, it is challenging to dock steroids in the correct orientation. The consensus among results obtained from various docking approaches and scoring functions may help to reduce the uncertainty.

Although antidins sbAvd-7–9 bind progesterone with nanomolar affinity, and Btn binds with high affinity, sbAvd-7–9 could be used in Btn-free assays, *e.g.* for steroids.

## 6.3 Multipurpose avidins

The search for novel Avds have come a long way since the 1940s when chicken Avd was newly discovered. Not only have novel Avds across prokaryotic/eukaryotic species been successfully identified, but various forms of engineered Avds have also been developed. With the characterization of bradavidin and chicken Avd-based antidins, we now have natural and artificial protein scaffolds possessing binding affinities for non-Btn ligands, *e.g.* steroids and the peptide ligand Brad-tag. Upon reaching the desired binding affinity, these scaffolds could be used to construct a circular mutant, dual chain/single chain form with, *e.g.* two high-affinity sites for one ligand and two other sites, for the second ligand, or multifunctional Avds with subunits having lowered or no affinity to Btn (Laitinen et al., 1999; Leppiniemi et al., 2011; Morag et al., 1996; Reznik et al., 1998; Takakura et al., 2013), or scAvds with 'dead' subunits that do not bind ligands (Howarth et al., 2006). In the future, these multipurpose Avds may have use in various branches of bio(nano)technology.

# 7. Conclusion

In this thesis, we present: i) crystal structures of a novel sbAvd, sbAvd-2 (I117Y) in apo [PDB:4U46] and progesterone-bound form [PDB:5LUR], and core-bradavidin–Btn complex [PDB:4BBO]; ii) models of Brad-tag containing Avds and steroid-binding sbAvd-7–9; and iii) computational binding studies on the modeled antidins with progesterone and Btn. Through these results, we probed the structural determinants responsible for binding Btn and, especially, non-Btn ligands in natural and artificial Avds.

In publication I, we successfully developed a number of antidins capable of binding steroids like progesterone and small molecules such as cholic acid with micromolar and nanomolar affinity. The crystal structure of apo sbAvd-2 (I117Y) reported in publication I proved the robustness of the antidin scaffold and helped further design of antidins. In manuscript II, we expanded our arsenal further by developing antidins binding specific steroids, for example, progesterone. The potency of a few selected antidins were tested using a microplate-based assay to detect progesterone in female dog serum samples in order to monitor breeding times. Thus, antidins have the potential to replace antibodies in diagnostics if higher affinities can be achieved. We also reported the crystal structure of the sbAvd-2 (I117Y) in complex with progesterone, which gave us critical insight into the progesterone binding mode of Arg13, Met14, Asn15 and His16 containing sbAvds. This structure also helped us build computational models for the high-affinity progesterone binders, sbAvd-7–9, to which we docked progesterone and Btn to study key interactions formed during ligand binding. A large number of the docked progesterone poses obtained from the docking complexes were inverted in comparison to the progesterone ligand in the crystal structure of sbAvd-2 (I117Y)–progesterone complex [PDB:5LUR]. This study underscored the shortcomings of ligand-docking programs when the structure has been modeled or does not reflect the binding conformation.

In publication III, we focused on bradavidin, a bacterial Avd from *Bradyrhizobium diazoefficiens* (Delamuta et al., 2013; Nordlund et al.,

2005b). Through the determination of the core-bradavidin–Btn complex crystal structure, we were able to study the ligand-binding mechanism of bradavidin with two ligands, Brad-tag and Btn. We constructed two variants of bradavidin: the core-bradavidin CC mutant used for testing the importance of the disulphide bridge in ligand binding, and the core-bradavidin V1 variant used for masking the exposed hydrophobic residues in core-bradavidin. Biophysical analyses of these variants did not reveal any significant differences in Btn-binding affinity in comparison to core-bradavidin, but the low yield of the CC mutant protein indicated a possible role of the disulphide bridge in protein folding; the role of the disulphide bridge for Brad-tag binding could not be excluded either. Models of two Brad-tag containing Avds – rhodavidin and Bradavidin A2 – were also built to observe their Brad-tag binding mode, which is unlikely to be different from wt bradavidin. Overall, the comparison of the wt and core-bradavidin structures, and the core-bradavidin variants, helped us to understand how Btn and the intrinsic peptide ligand could both bind to an Avd scaffold.

In the future, through the identification of novel Avds from phylogenetic studies including more and more examples from different species, and by the construction of additional high-affinity steroid-binding and other non-Btn ligand-binding antidins, we should be able to develop high-affinity multipurpose dc/sc Avds, capable of binding varied ligands simultaneously and henceforth be able to propel the Avd-Btn technology even further.

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