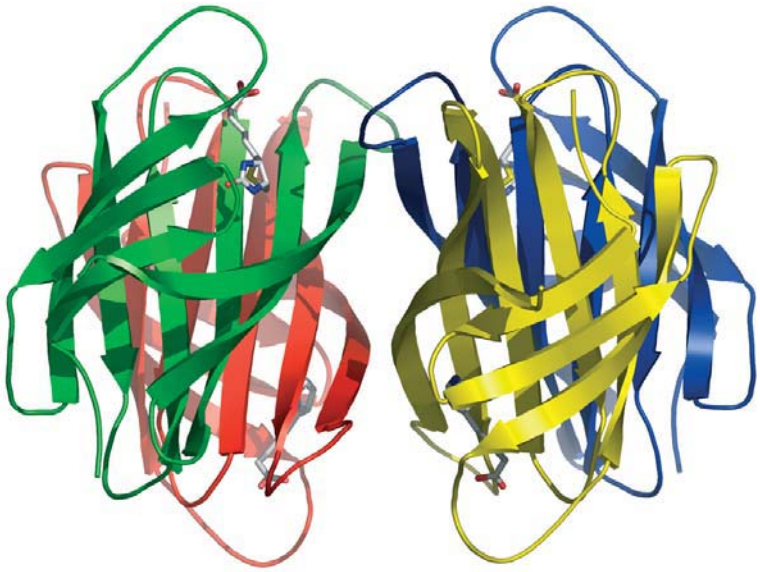
JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
153

Vesa Hytönen

The Avidin Protein Family

Properties of Family Members and Engineering of Novel Biotin-Binding Protein Tools



JYVÄSKYLÄN YLIOPISTO

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2005

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Editors
Jukka Särkkä
Department of Biological and Environmental Science, University of Jyväskylä
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Cover picture: Three-dimensional structure of avidin-related protein 4/5 determined with X-ray crystallography (PDB 1y55). The subunits of the tetrameric protein are coloured and secondary structure elements are indicated by cartoons. Bound ligands, four molecules of D-biotin, are shown in sticks. The figure is generated using PyMOL. Picture by Vesa Hytönen.

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ABSTRACT

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Diss.

Chicken avidin family consists of several proteins which bind a small vitamin, D-biotin. One of these proteins, avidin, is a widely used biological tool in the life sciences. The other members of the family are avidin-related proteins (AVRs). Avidin is thought to be an antimicrobial protein whereas the biological role of AVRs is not known. AVRs have been characterised only as recombinant proteins.

The aim of this study was to characterise the structural and functional features of AVRs, especially the identical proteins coded by avidin related genes 4 and 5 (AVR4/5). Another objective was to develop new tools for avidin-biotin technology.

In order to enable bacterial avidin production, the original avidin signal peptide was replaced with a secretion signal from a bacterial protein. The avidin produced in *Escherichia coli* was isolated and purified using affinity chromatography. In its properties, the produced avidin resembled avidin extracted from chicken egg-white, but it lacked glycosylation. Successful *E. coli* expression provided the possibility to economically produce avidin and avidin-like proteins for research and applications.

Mutagenesis of a C-terminal cysteine residue of AVR4/5 to serine changed its oligomerization properties without affecting those of biotin-binding or thermostability. We were able to substitute part of the avidin sequence with a sequence stretch from AVR4/5. The chimeric protein thus obtained showed increased thermostability when compared to that of avidin. The high-resolution structure of AVR4/5 was determined by X-ray crystallography. The higher thermal stability of AVR4/5 is the result of only slight changes in the structural elements compared to avidin.

The novel proteins obtained in this study may offer tools for avidin-biotin technology and also improve the understanding of biotin-binding process.

Key words: Avidin; avidin-related proteins; bacterial expression; biotin; protein engineering; protein structure.

V. Hytönen, University of Jyväskylä, Department of Biological and Environmental Science, P. O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address Vesa Hytönen
Department of Biological and Environmental Science
P. O. Box 35, FI-40014 University of Jyväskylä, Finland
veshyto@bytl.jyu.fi

Supervisor Professor Markku Kulomaa
Department of Biological and Environmental Science
University of Jyväskylä, Finland

Present address:
Institute of Medical Technology
University of Tampere, Finland

Reviewers Professor Pentti Tuohimaa
Department of Medicine
University of Tampere, Finland

Professor Kari Keinänen
Department of Biological and Environmental Sciences
University of Helsinki, Finland

Opponent Professor Hans Söderlund
VTT Biotechnology
VTT, Finland

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which are referred to in the text by their Roman numerals. Some unpublished results are also presented.

I Hytönen, V. P., Laitinen, O. H., Airenne, T. T., Kidron, H., Meltola, N. J., Porkka, E. J., Hörhä, J., Paldanius, T., Määttä, J. A., Nordlund, H. R., Johnson, M. S., Salminen, T. A., Airenne, K. J., Ylä-Herttuala, S. & Kulomaa, M. S. 2004. Efficient production of active chicken avidin using a bacterial signal peptide in *Escherichia coli*. Biochem. J. 384: 385–390.

II Hytönen, V. P., Nyholm, T. K., Pentikäinen, O. T., Vaarno, J., Porkka, E. J., Nordlund, H. R., Johnson, M. S., Slotte, J. P., Laitinen, O. H. & Kulomaa, M. S. 2004. Chicken avidin-related protein 4/5 shows superior thermal stability when compared with avidin while retaining high affinity to biotin. J. Biol. Chem. 279: 9337–9343.

III Eisenberg-Domovich, Y., Hytönen, V. P., Wilchek, M., Bayer, E. A., Kulomaa, M. S. & Livnah, O. 2005. Crystal structure of an avidin-related protein: Insight into high-affinity biotin binding and protein stability. Acta Crystallogr. D, 61: 528–538.

IV Hytönen, V. P., Määttä, J. A., Nyholm, T. K. M., Livnah, O., Eisenberg-Domovich, Y., Hyre, D., Nordlund, H. R., Hörhä, J., Niskanen, E. A., Paldanius, T., Kulomaa, T., Porkka, E. J., Stayton, P. S., Laitinen, O. H. & Kulomaa, M. S. 2005. Design and construction of highly stable, protease-resistant chimeric avidins. J. Biol. Chem. 280: 10228–10233.

MY RESPONSIBILITIES IN THE THESIS ARTICLES

Article I The study was planned in collaboration with Dr. Olli Laitinen. I was mainly responsible for the practical work relating to this study. Olli also significantly participated in the writing of the article.

Article II I was mainly responsible for the planning, practical work and writing of this study. Dr. Thomas Nyholm carried out the calorimetric analyses. Dr. Olli Pentikäinen did the molecular modelling. Dr. Olli Laitinen participated to the writing of the article.

Article III Yael Eisenberg-Domovich and Professor Oded Livnah are responsible for the experimental analysis of the proteins. I participated in the planning of the work, protein production and writing of the article. Jarno Hörhä and Eevaleena Porkka performed part of the practical work related to protein production under my supervision.

Article IV I was mainly responsible for the planning, practical work and writing up of this study. Juha Määttä, Eevaleena Porkka and Jarno Hörhä performed a significant part of the practical work under my supervision. The calorimetric analyses were done by Dr. Thomas Nyholm. Tuomas Kulomaa performed the radiobiotin dissociation analyses under the supervision of Dr. David Hyre and myself. Professor Oded Livnah and Yael Eisenberg-Domovich participated in the structural analysis of the proteins.

ABBREVIATIONS

3D	three-dimensional
ACC	acetyl CoA carboxylase
AVR	avidin-related gene
AVR	*AVR* encoded protein
Avd	avidin
BAP	biotin-accepting peptide
BBP	biotin-binding protein
BCCP	biotin carboxyl carrier protein
BNP	biotinyl p-nitrophenyl ester
BPL	biotin protein ligase
BSA	bovine serum albumin
ChiAVD	chimeric avidin mutant
C_p	heat capacity
ΔC_{pL}	change in heat capacity upon ligand binding
DSC	differential scanning calorimetry
dcAvd	dual chain avidin
FPLC	fast protein liquid chromatography
GCC	geranoyl-CoA carboxylase
GCD	glutaconyl-CoA decarboxylase
H	enthalpy
$H^{\ddagger}$	activation enthalpy
h	Planck's constant
HABA	2-(4'-hydroxyazobenzene) benzoic acid
HCS	holocarboxylase synthetase
ITC	isothermal titration calorimetry
K_a	association constant
k_{ass}	association rate constant
k_B	Boltzmann's constant
K_d	dissociation constant
k_{diss}	dissociation rate constant
kDa	kilodalton
K_T	Michaelis-Menten constant for carrier-mediated uptake
LB	Luria-Bertani
MCC	methyl-crotonyl carboxylase
MMD	methylmalonyl-CoA decarboxylase
MRI	magnetic resonance imaging
OxD	oxaloacetate decarboxylase
PAC	pyruvic acid carboxylase
PBMCs	peripheral blood mononuclear cells
PCC	propionyl CoA carboxylase
PCR	polymerase chain reaction
PDB	protein data bank
pI	isoelectric point

PNA	peptide nucleic acid
R	universal gas constant
S	entropy
$S^{\ddagger}$	activation entropy
SBP	seed biotinylated protein
scAvd	single chain avidin
SDS-PAGE	sodium-dodecyl sulphate polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
T7	bacteriophage T7
TC	transcarboxylase
T_m	transition midpoint of heat denaturation
T_r	transition midpoint of oligomeric disassembly
UC	urea carboxylase
UV	ultraviolet
wt	wild type
Å	ångström (0.1 nm)

1 INTRODUCTION

The negative effects, impressive changes in skin and hair, leading to emaciation and finally death, caused by egg-white used as a food (Steiniz 1898, Boas 1927) led to the discovery of a new vitamin, biotin (Gyorgy 1939, Gyorgy et al. 1940). Egg-white was found to contain a special agent causing "egg-white injury" in animals by harvesting biotin (Eakin et al. 1940a, Eakin et al. 1940b). This agent, a biotin-binding protein, was isolated and partially purified for first time in 1941, and named avidin since it has *avid*ity towards biot*in* (Eakin et al. 1941).

Chicken egg-white protein avidin and the analogous protein streptavidin from the bacteria *Streptomyces avidinii* have become common tools in the life sciences (Wilchek & Bayer 1990a, Wilchek & Bayer 1999). Several modified forms of (strept)avidin have been generated and characterised (Chilkoti et al. 1995a, Sano & Cantor 1995, Reznik et al. 1996, Sano et al. 1997, Marttila et al. 1998, Freitag et al. 1999b, Hyre et al. 2000, Marttila et al. 2000, Laitinen et al. 2003, Marttila et al. 2003, Nordlund et al. 2003a, Nordlund et al. 2003b). Although avidin is the most widely known biotin-binding protein in chicken, there are several other avidin-related genes in the chicken genome (*AVR*-genes) (Keinänen et al. 1988, Keinänen et al. 1994, Ahlroth et al. 2000, Ahlroth et al. 2001a, Ahlroth et al. 2001b, Niskanen et al. 2005). These genes seem to carry functional promoter regions and the mRNA of some of them have been detected in chicken tissues during inflammation (Kunnas et al. 1993). In order to study the properties of the putative proteins they code, avidin-related proteins (AVRs) were recently expressed as recombinant proteins (Laitinen et al. 2002).

In this study, we generated an efficient method of producing avidin in bacteria (I). This system facilitates the avidin research, by making avidin faster and easier to produce. We also worked with another member of the avidin family, namely avidin related protein 4/5, which is the identical product of genes *AVR4* and *AVR5*. We found this protein to have extremely high thermostability and to bind biotin almost as tightly as avidin (II). The three-dimensional structure of AVR4/5 revealed that only slight differences in its structure are responsible for the differences in its functional properties when

compared to those of avidin (III) Furthermore, we used the methods of molecular biology to generate chimeric forms of avidin and AVR4/5. These modified avidins showed encouraging properties and also supported the hypothesis of the importance of a particular mutation in a specific subunit interface of AVR4/5 (IV). These novel avidin forms offer powerful tools for avidin-biotin technology and also provide insights into the fine-tuning of protein function between similar proteins.

The properties of (strept)avidin have thoroughly been discussed in several previous reviews of the literature in PhD dissertations produced by our group (Airenne 1998, Laitinen 2001, Marttila 2003, Nordlund 2003). To expand understanding of this system, the emphasis in the following review of the literature is on other molecules interacting with biotin in cells.

2 REVIEW OF THE LITERATURE

Biotin, vitamin H, is an important factor in biochemical and cellular processes. D-biotin is a small water-soluble molecule with a molecular weight of 244.2 g/mol (Fig. 1). The attachment of biotin, biotinylation, regulates the activity of enzymes involved in the central metabolism of the cell. The attached biotin serves as a carrier of an activated carboxyl group in carboxylation, decarboxylation and transcarboxylation reactions (Chapman-Smith & Cronan 1999b, McMahon 2002). Biotin is also known to regulate the expression of various genes, namely glucokinase (Dakshinamurti & Litvak 1970, Chauhan & Dakshinamurti 1991), biotin-dependent carboxylases and holocarboxylase synthetase (Rodriguez-Melendez et al. 2001, Solorzano-Vargas et al. 2002), biotin transporters (Manthey et al. 2002, Crisp et al. 2004), cytokines (Zempleni et al. 2001, Manthey et al. 2002, Rodriguez-Melendez et al. 2003) and oncogenes N-myc, c-myb, N-ras and raf (Scheerger & Zempleni 2003). Preliminary results have shown that biotin supplementation affects the expression of over 200 of the 12000 tested human genes (Rodriguez-Melendez & Zempleni 2003). It has been found that the histones involved in the packing of DNA are also biotinylated (Hymes et al. 1995, Hymes & Wolf 1999, Stanley et al. 2001, Manthey et al. 2002, Peters et al. 2002, Scheerger & Zempleni 2003, Crisp et al. 2004). There are known diseases caused by the malfunction of the biotin processing machinery (Pacheco-Alvarez et al. 2002 and references therein). The recent success in the study of biological processes linked to biotin has evoked significant interest in this field, making biotin an interesting research topic.

From the viewpoint of molecular biology, the proteins linked to the biotin are the greatest interest. Some of the proteins related to biotin metabolism are characterised in detail and the three-dimensional structures of these proteins are also known. The function of the protein is always reflected in its structure and *vice versa*, and by comparing the existing structures one can learn to understand the mechanism of biological recognition and function. Biotin protein ligase (BPL) and its bacterial analogue BirA is a protein specifically binding biotin, and this protein attaches biotin to other proteins (Chapman-

Smith & Cronan 1999a). The biotin carboxyl carrier protein (BCCP) is a part of the acetyl coenzyme A carboxylase complex carrying the biotin cofactor and a substrate of BPL (Athappilly & Hendrickson 1995, Jitrapakdee & Wallace 2003). Biotinidase is a protein which releases the biotin from biotinylated proteins (Hymes & Wolf 1999). Oviparous vertebrates have a characteristic biotin-binding protein in their egg-white, avidin (Green 1975). The function of this protein is thought to be only to harvest the biotin from the environment and therefore prevent the growth of competing organisms (Green 1975, Korpela et al. 1981), i.e., prevent the proteins mentioned above from functioning. All these proteins are tightly coupled to this small molecule, biotin.

The interaction between biotin and (strept)avidin is extremely tight, and this property has been employed for various purposes. Beyond its uses in biotechnology and pharmacy, biotin is a vitamin molecule which has important functions in various cellular processes.

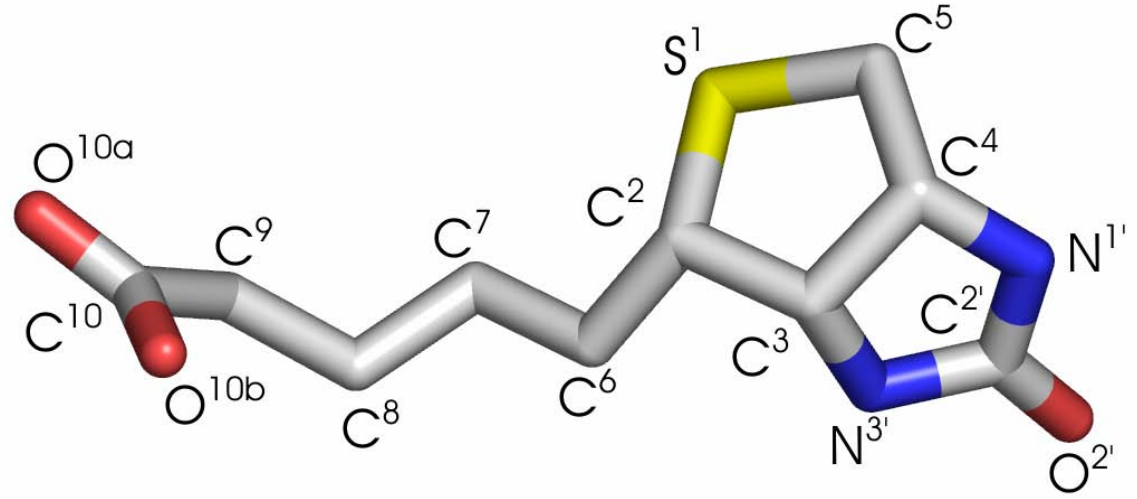

FIGURE 1 Structure of D-biotin. The coordinates of biotin are from the avidin-biotin complex (PDB 2avi). The numbering of atoms is according to DeTitta et al. (1976).

2.1 Introduction to biotin biology

Biotin is synthesised in a multi-step process, as presented in Fig. 2 (Streit & Entcheva 2003). The genes involved in biotin synthesis are organised in operons in many organisms. Most microbes, plants and fungi are able to synthesise biotin themselves [reviewed in Streit & Entcheva (2003)]. Most *Archaea* cannot, according to genetic comparisons, probably perform biotin synthesis (Streit & Entcheva 2003). Analogously, many eukaryotic organisms, including human, need to obtain a sufficient amount of biotin from food. The recommended dietary intake of biotin is 30 µg/day for humans (Mock et al. 2003).

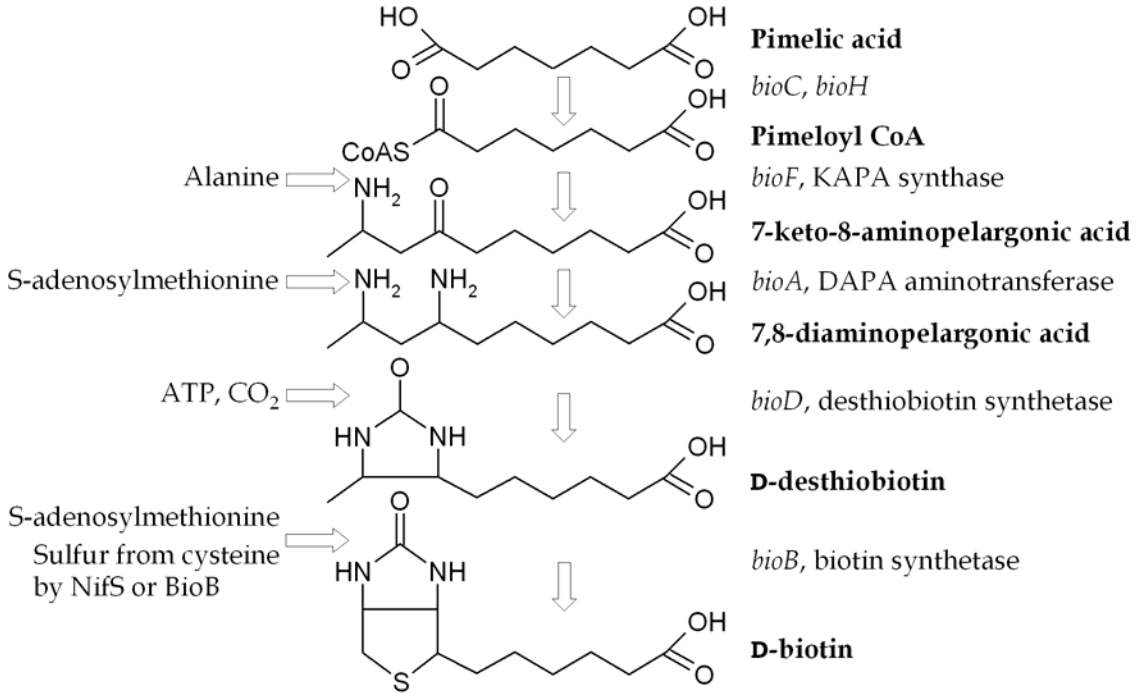

FIGURE 2 Overview of biotin synthesis. The genes and proteins involved in the synthesis steps in gram-negative bacteria are shown on the right. The figure is adapted from Streit & Entcheva (2003).

The concentration of biotin in normal human plasma is 0.5 nM (Mardach et al. 2002). Lack of biotin in the diet results in changes in the skin and hair in mammals. This is followed by emaciation, finally leading to death (Gyorgy 1939). Biotin deficiency is known to cause a rapid decline of carboxylase activities in humans (Manthey et al. 2002). Pyruvate carboxylase is a key enzyme in gluconeogenesis and, therefore, lack of biotin will lead to fasting hypoglycaemia and ketosis (Bender 1999). This has been suggested to be a link between low biotin status and infant death (Johnson et al. 1980). Acetyl-CoA carboxylase is an important enzyme in fatty acid synthesis and biotin deficiency can affect the acetate metabolism *via* this protein. Relation between fatty acid elongation and biotin deficiency has been showed by Kopinski et al. (1989). Propionyl-CoA carboxylase catalyses reactions linked to the metabolism of several molecules, including isoleucine and methionine. Therefore, lack of biotin leads to urinary excretion of propionate (Bender 1999). Impairement of Methylcrotonyl-CoA carboxylase activity leads to urinary excretion of methylcrotonate. 3-Hydroxyisovalerate is an onward metabolite of methyl-crotonate. A recent study showed that the increased 3-hydroxyisovalerate excretion frequently observed in pregnant women (Mock & Stadler 1997, Mock et al. 1997) is in fact linked to biotin deficiency (Mock et al. 2002). There are known problems associated with biotin deficiency in mice babies, such as cleft palate, microglossia and micromelia (Watanabe & Endo 1984). Studies with turkeys have shown reduced hatchability due to biotin deficiency (Robel 1987). It has been suggested that marginal biotin deficiency is teratogenic also in humans (Zempleni & Mock 2000). Another study provided evidence that biotin

deficiency decreases the life span and fertility in *Drosophila melanogaster*. It was also found to be associated with decreased resistance to oxidative stress. Furthermore, male flies were found to be more susceptible than females to developing biotin deficiency (Landenberger et al. 2004).

Biotin is covalently linked to biotin-dependent enzymes. Therefore, there is need for a protein that can release biotin from degraded proteins. Biotinidase specifically catalyses this reaction and makes biotin available for reuse. Analogously, biotin in food is often linked covalently to other molecules and need to be released by biotinidase. This is an important reaction, since failures in biotinidase function cause biotin deficiency (Hymes & Wolf 1999). The localisation of biotinidase (EC 3.5.1.12) is not well known. In addition to biotinidase in plasma, which is thought to be secreted from the liver (Pispa 1965, Weiner et al. 1983), at least one intracellular form of biotinidase, probably locating in microsomes and lysosomes seems also exist (Stanley et al. 2004).

Biotin is transported inside the cell using a biotin transporter. Several studies have shown that biotin is transported from plasma into the liver by Na^+-coupled, electroneutral, saturable and structurally specific mechanism. The reported Michaelis-Menten constants for carrier-mediated uptake (K_T) are around 100 µM (Said et al. 1990, Weiner & Wolf 1990, Weiner & Wolf 1991, Said et al. 1992, McCormick & Zhang 1993, Said et al. 1994). In humans, two candidates for the biotin transporter have been described. A multivitamin transporter, named SMVT, is known to be able to transport panthothenic acid and lipoic acid in addition to biotin. SMVT is cloned from human JAR choriocarcinoma cells (Prasad et al. 1998) and from human Caco-2 intestial cell line (Wang et al. 1999). These transporters showed K_T values for biotin of 15 µM and 3 µM, respectively. Another biotin transporter has been found in peripheral blood mononuclear cells (PBMCs). This transporter has a significanly lower K_T (2.6 nM) for biotin compared to SMVT (Zempleni & Mock 1998). Furthermore, biotin transport is not inhibited by pantothenic acid in the case of this form (Zempleni & Mock 1999). Sucrose-H^+ symporter AtSUC5 has been found to transport biotin in plant cells (Ludwig et al. 2000). Rogers and Lichstein (1969) have described a carrier-mediated high-affinity (K_T = 0.3 µM) biotin uptake mechanism in *Saccharomyces cerevisiae*. The gene coding for this transporter has been identified and named *VHT1* (Stolz et al. 1999). A functionally similar gene, *SpVht1p*, was also found in *Schizosaccharomyces pombe*, showing only slight sequence homology with *VHT1* (Stolz 2003). In bacteria, at least two decarboxylases working as a biotin transporters have been characterised: Methylmalonyl-CoA decarboxylase (Galivan & Allen 1968) and glutaconyl-CoA decarboxylase (Buckel & Semmler 1983). *E. coli* takes up biotin by a carrier-mediated, high affinity (K_T = 0.14 µM) process (Prakash & Eisenberg 1974). Recently, *E. coli* and also other gram-negative bacteria, *Salmonella enterica* and *Pseudomonas aeruginosa*, have been found to transport up to 31-residue biotinylated peptides *via* a biotin transporter (Walker & Altman 2005).

The biotin cycle in cells and the proteins interacting with biotin are schematically presented in Fig. 3.

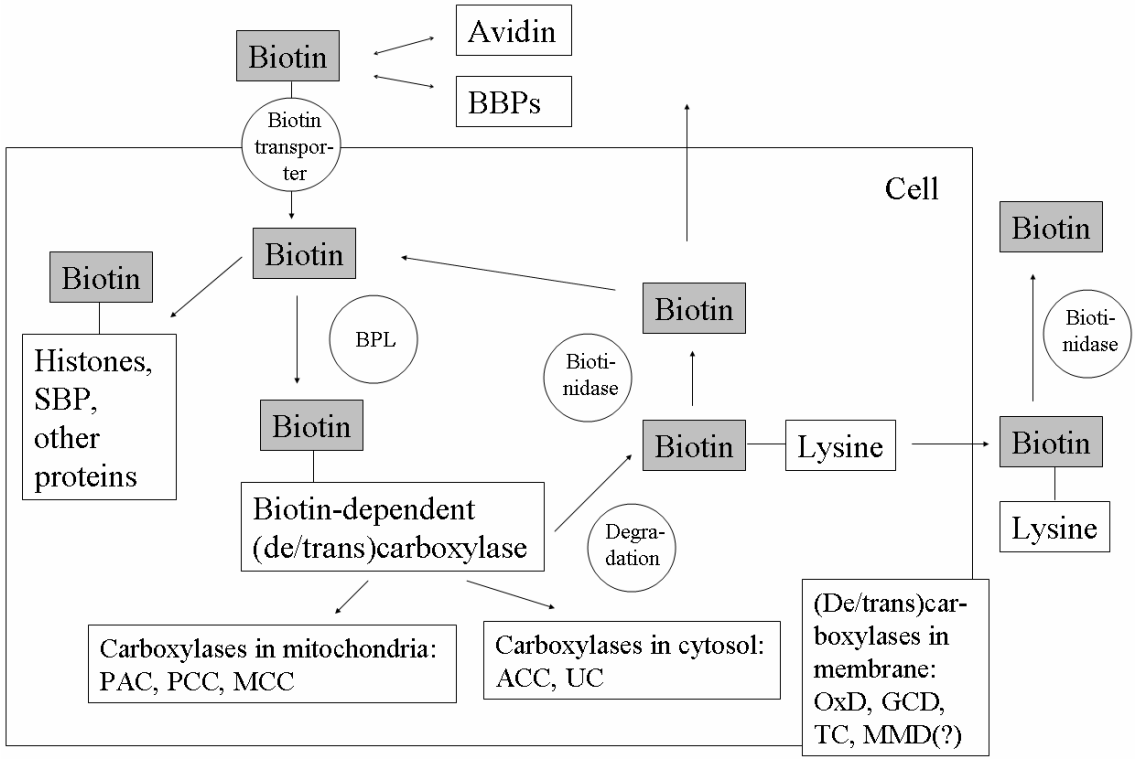

FIGURE 3 A schematic picture of the protein network interacting with biotin in different types of cells. Schematic presentation shows enzymes carrying particular reactions inside circles (BPL, biotin protein ligase). One should note that avidin and yolk biotin-binding proteins (BBPs) are known to exist only in oviparous vertebrates (Korpela et al. 1981). Seed biotinylated proteins (SBPs) exist only in plants (Duval et al. 1994a). The abbreviations for biotin-dependent carboxylases shown as examples in the figure are pyruvic acid carboxylase (PAC; EC 6.4.1.1), propionyl CoA carboxylase (PCC; EC 6.4.1.3), methyl-crotonyl carboxylase (MCC; EC 6.4.1.4) and acetyl CoA carboxylase (ACC; EC 6.4.1.2) (Gamachi et al. 2003). There are two ACC isoforms found in plants, one localizing in cytoplasm and another in plastids (Alban et al. 1994). Geranoyl-CoA carboxylase (GCC; EC 6.4.1.5) exists in some bacteria and is thought also to be present in plant plastids (Nikolau et al. 2003). Urea carboxylase (UC; EC 6.3.4.6) is known to be present in yeast species and green algae but not in plants (Roon et al. 1972, Roon & Levenberg 1972). Methylmalonyl-CoA carboxytransferase, also called transcarboxylase, (TC; EC 2.1.3.1) transfers a carboxyl group from methylmalonyl-CoA to pyruvate to form propionyl-CoA and oxalacetate. TC from *Veillonella alcalescens* has been characterised (Hoffmann et al. 1989). Oxaloacetate decarboxylase (OxD; EC 4.1.1.3) has been characterised in *Klebsiella aerogenes* and decarboxylates glutaconyl-CoA and methylmalonyl-CoA. The process is accompanied by the extrusion of two sodium ions from cells. Methylmalonyl-CoA decarboxylase (MMD; EC 4.1.1.41) acts as a biotin-dependent sodium pump. The MMD is thought to be located in the cell membrane (Galivan & Allen 1968). The glutaconyl-CoA decarboxylase (GCD; EC 4.1.1.70) from *Acidaminococcus fermentans* is a biotinyl-protein, requires Na^+, and acts as a sodium pump (Buckel & Semmler 1983).

2.2 Attaching of biotin to proteins

The analysis of the biotin-interacting proteins allows understanding of the system involved in biotin biology. The following chapters introduce the structural properties of the biotin-binding proteins characterised so far. The biotin-binding mechanism of these proteins with their determined X-ray crystallographic structures are also presented in detail and compared to each other.

2.2.1 Biotin holoenzyme synthetase/bio repressor

Biotin protein ligase (BPL) works as an enzyme covalently attaching biotin to acceptor proteins. This protein catalyses both the activation of biotin by ATP and the attachment of the biotinyl-5'-AMP to the target protein (Chapman-Smith et al. 2001). There is usually only a single BPL in each organism (Kim et al. 2004), *Arabidopsis thaliana* being the only known exception, containing both a cytoplasmic and a putative chloroplast form of the enzyme (Denis et al. 2002). The BPL in mammals is called holocarboxylase synthetase (HCS). In *E. coli*, the analogous protein is biotin holoenzyme synthetase/bio repressor (BirA), which is a 321 residue (33.5 kDa) protein working both as a biotin operon repressor as well as an enzyme, namely biotin[acetyl-CoA carboxylase]synthetase (Eisenberg et al. 1982). BirA is thought to work as a dimer (Weaver et al. 2001). The three-dimensional structure of *E. coli* BirA has been determined (Wilson et al. 1992) (Fig. 4). BirA is organized in three domains. The N-terminal domain (residues 1–60) contains three alpha-helices, which are packed against two strands of an antiparallel beta-sheet (Wilson et al. 1992). This domain contains a helix-turn-helix motif, which participates in DNA binding. The central domain of BirA consists of five alpha-helices and a mixed seven-stranded beta-sheet. One face of this beta-sheet is exposed to solvent and this area forms the active site of the protein, i.e. the biotin-binding site. There are four loops in the central domain, which do not appear in electron density maps. This may be due to the mobility of the loops. One of the loops (residues 116-124) contains sequence GRGRRG, which includes an ATP binding consensus sequence (Wierenga et al. 1986). The C-terminal domain of BirA consists of two three-stranded antiparallel beta-sheets. These strands form a beta-sandwich (Wilson et al. 1992) (Fig. 4).

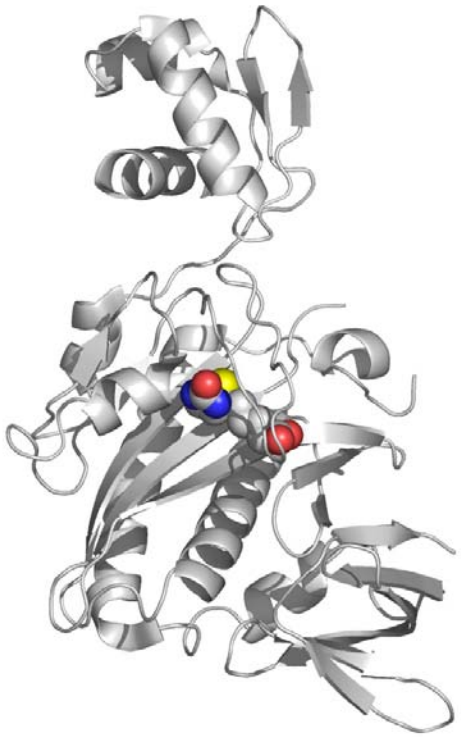

FIGURE 4 Overall structure of BirA (Weaver et al. 2001). The structure of the BirA with bound biotin was determined by X-ray crystallography (PDB 1hxd).

In *E. coli*, the only characterised target for biotinylation by BirA, is the biotin carboxyl carrier protein (BCCP) in acetyl-CoA carboxylase. In other organisms, a small number of different carboxylases, decarboxylases and transcarboxylases are biotinylated by BPL; these are summarised in Fig. 3. A recent study suggested that BPL is involved in histone biotinylation in humans (Narang et al. 2004). It has also been found that several biotinylated proteins can be isolated by streptavidin-affinity chromatography from mammalian cells (de Boer et al. 2003). Moreover, this research group also detected histones in the group of isolated proteins. Furthermore, de Boer et al. were also able to characterise the isolated proteins by mass spectrometry, identifying many novel targets for biotinylation, such as RNA processing proteins, in addition to known biotinylated proteins. Notably, Kim et al. were recently able to identify a novel biotinylation target from *Saccharomyces cerevisiae*, Arc1p, which is employed in RNA processing (2004). The biotinylation reaction is presented in Eq. 1 and Eq. 2.

$$\text{Biotin} + \text{ATP} \leftrightarrow \text{biotinyl-5'-AMP} + PP_i \qquad \text{Eq. 1}$$

$$\text{Biotinyl-5'-AMP} + \text{protein} \leftrightarrow \text{biotinyl-protein} + \text{AMP} \qquad \text{Eq. 2}$$

2.2.2 Biotin carboxyl carrier protein

Biotinylation of the biotin-dependent enzyme complex by BPL is targeted to the C-terminal part of the polypeptide or to a specific subunit called the biotin carboxyl carrier protein (BCCP), which forms a part of the complex (Samols et

al. 1988). Biotinylation usually occurs at around 35 residues from the C-terminus of the protein (Clarke et al. 2003). Many BPLs have been found to be able to biotinylate biotin-acceptor domains from other species (Cronan & Reed 2000). In the acetyl-CoA carboxylase of *E. coli*, BCCP has a molecular weight of 16.7 kDa (Fall & Vagelos 1972). The full-length form of BCCP is proteolytically processed to a stable short form (Fall et al. 1971, Fall & Vagelos 1972, Fall & Vagelos 1975) consisting of the C-terminal biotinylation domain and N-terminal domain , which is involved in subunit interaction. The functional form of BCCP is thought to be a dimer (Fall & Vagelos 1972). A recent study showed that two BCCP molecules form a complex with one biotin carboxylase in the *E. coli* acetyl-CoA carboxylase enzyme assembly (Choi-Rhee & Cronan 2003). Isolated BCCP forms large aggregates in solution (Nenortas & Beckett 1996).

Biotinylation occurs at a lysine residue located in a conserved sequence, AMKM (Samols et al. 1988, Cronan & Reed 2000). The adjacent methionine before biotinylated lysine residue is very strongly conserved and methionine also occurs after lysine in most known biotinylation sites (Duval et al. 1994b, Thelen et al. 2001). However, in the case of plants and cyanobacteria the sequence is MKL (Thelen et al. 2001). The known deviations from this MKM/MKL rule are Arc1p in *Saccharomyces cerevisiae*, which is biotinylated in the lysine residue middle of sequence SKD (Kim et al. 2004) and SBP65, in which biotinylation occurs at sequence GKF (Duval et al. 1994b).

The structure of the C-terminal fragment of BCCP from *E. coli* has been determined using both X-ray crystallography and NMR (Athappilly & Hendrickson 1995, Blanchard et al. 1999, Roberts et al. 1999). The protein consists of two sets of four antiparallel beta strands. The structure is stabilized by hydrophobic core residues. There is only one cysteine residue in BCCP, which is a part of the hydrophobic core (Athappilly & Hendrickson 1995). Each subunit of BCCP binds one biotin molecule, which is covalently attached to the lysyl residue in position 122, forming biocytin located at a beta turn (Fig. 5). The structural characterisation of 1.3S subunit of transcarboxylase from *Propioni-bacterium shermanii* revealed an almost identical structure of a biotin-accepting C-terminal domain (Reddy et al. 1998). However, the protein form *P. shermanii* lacks the extension called the "protruding thumb" seen in the BCCP from *E. coli* (on the left in Fig. 5 close to bound biotin).

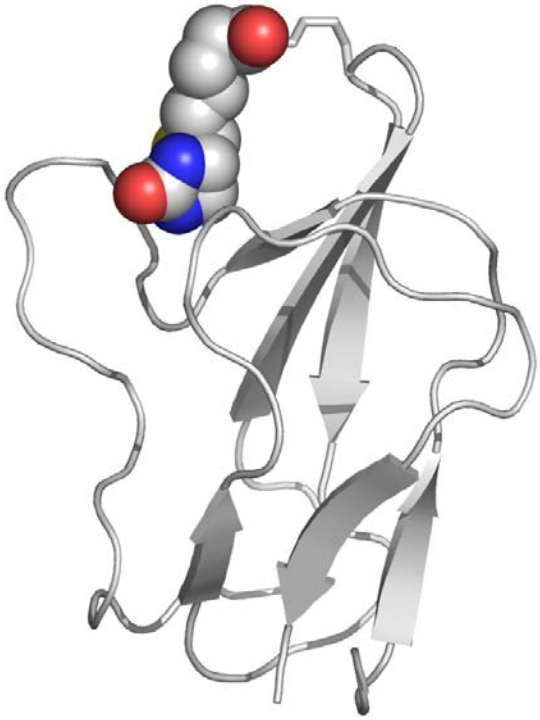

FIGURE 5 Structure of BCCP with bound biotin (PDB 1bdo). X-ray crystallographic structure was determined by Athappilly & Hendrickson (1995).

BCCP is a part of the acetyl-CoA carboxylase complex in *E. coli*. It is both carboxyl acceptor and donor in transcarboxylase reactions catalyzed by this complex (Fall & Vagelos 1972). Acetyl CoA carboxylase is present in all animals, plants and bacteria. The transcarboxylase reaction is one step in the biosynthesis of long-chain fatty acids. The enzyme complex biotin-dependently carboxylates acetyl-CoA to form malonyl-CoA (Lane et al. 1974, Wakil et al. 1983). The transcarboxylation reaction consists of the following steps (Wakil et al. 1983):

$$\text{Enzyme–biotin} + \text{MgATP} + HCO_3^- \leftrightarrow \text{enzyme–biotin–}CO_2^- + \text{MgADP} + P_i \qquad \text{Eq. 3}$$

$$\text{Enzyme–biotin–}CO_2^- + \text{acetyl–CoA} \leftrightarrow \text{malonyl–CoA} + \text{enzyme–biotin} \qquad \text{Eq. 4}$$

2.2.3 Seed biotinylated protein

It has been found that there is a special biotinylated 65 kDa protein in pea seeds, known as seed biotinylated protein (SBP65). This protein is absent from leaves, roots, stems, pods and flowers. Furthermore, the expression of the protein seems to be highest at the time of seed maturation, while the amount was found to decrease rapidly after seed planting. The protein exists as a multimer with a molecular weight of over 400 kDa, and consists of six to eight subunits. SBP65 is thought to function as a biotin store for the developing embryo (Duval et al. 1994a). Furthermore, a role as a regulator in the development process leading to the resting state of the embryonic cells has been suggested for SBP65 (Dehaye et al. 1997).

The gene encoding SBP65 has been cloned previously (Dehaye et al. 1997). This protein covalently binds one biotin per subunit (Duval et al. 1994a). The biotinylation domain of SBP65 is found to differ markedly from those of biotin enzymes (Duval et al. 1994b). Biotinylated protein analogous to SBP65 is also thought to exist in the soybean (Dehaye et al. 1997), and putative protein analogs have been identified in castor seeds (Roesler et al. 1996) and in carrot embryos (Wurtele & Nikolau 1992). SBP65 expressed recombinantly in *E. coli* has not been found to be biotinylated (Dehaye et al. 1997).

2.3 Structure and function of other biotin-binding proteins

In addition to BPL and the biotin carrier proteins, there are several other proteins known to interact specifically with biotin. Avidin and streptavidin are known from their use in life sciences. The other biotin-binding proteins have been less studied.

2.3.1 Avidin and streptavidin

Avidin is a homotetrameric glycoprotein found in chicken (*Gallus gallus*) egg-white. Each subunit consists of 128 amino acid residues and one glycomoiety attached to Asn17. The molecular weight of avidin is about 62 kDa. Avidin is a basic protein with an isoelectric point at pH 10.5. Each subunit has two cysteine residues forming a disulphide bridge inside the subunit. There are two methionines and four tryptophans per subunit in avidin (Green 1975).

Streptavidin is a bacterial protein from *Streptomyces avidinii*. It is homologous in structure with chicken avidin. However, there are several differences in its primary structure characteristics (Fig. 6) when compared to those of avidin (Argarana et al. 1986). The polypeptide chain, which is a subunit of the streptavidin tetramer, consists of 159 residues. However, this full-length polypeptide is often processed both C- and N-terminally to a core form consisting of 125–127 residues (Weber et al. 1989). Streptavidin lacks glycosylation and it is a slightly acidic protein (Green 1975). Core streptavidin does not have cysteines or methionines in sequence and there are six tryptophan residues per subunit (Chaiet & Wolf 1964).

Avidin and streptavidin are both very stable proteins. Thermal denaturation melting points (T_m) of 83-85 °C have been determined for avidin (Gonzalez et al. 1999, Nordlund et al. 2003a). Avidin is stable at pH 2-13 (Green 1975). Both avidin and streptavidin proteins are further stabilized by biotin binding. The determined T_m of the avidin-biotin complex is around 118 °C (Gonzalez et al. 1999, Nordlund et al. 2003a). The avidin-biotin-complex has been found to resist 9 M urea, 2% solutions of detergents Tween 20, Tween 40, Triton X-100, Zwittergent® 3-14 and Zwittergent® 3-08 as well as heating to 95.6 °C for five minute in the presence of these solutions excluding

Zwittergent® 3-14 (Ross et al. 1986). T_m of streptavidin has been found to be 75–84 °C (Weber et al. 1994, Gonzalez et al. 1999, Waner et al. 2004). The melting point of the streptavidin-biotin complex has been found to be around 112 °C (Gonzalez et al. 1999). Biotinylated bovine serum albumin exists as a complex with streptavidin Sepharose in 8 M guadinium hydrochloride (pH 1.3), 0.5 M HCl (pH 0.3), 2% SDS, 0.5 M NaOH (pH 13.7) as well as after heating for 15 minutes to 96 °C in the presence of these reagents (Rybak et al. 2004). The denaturation of avidin and streptavidin is an irreversible process under most conditions.

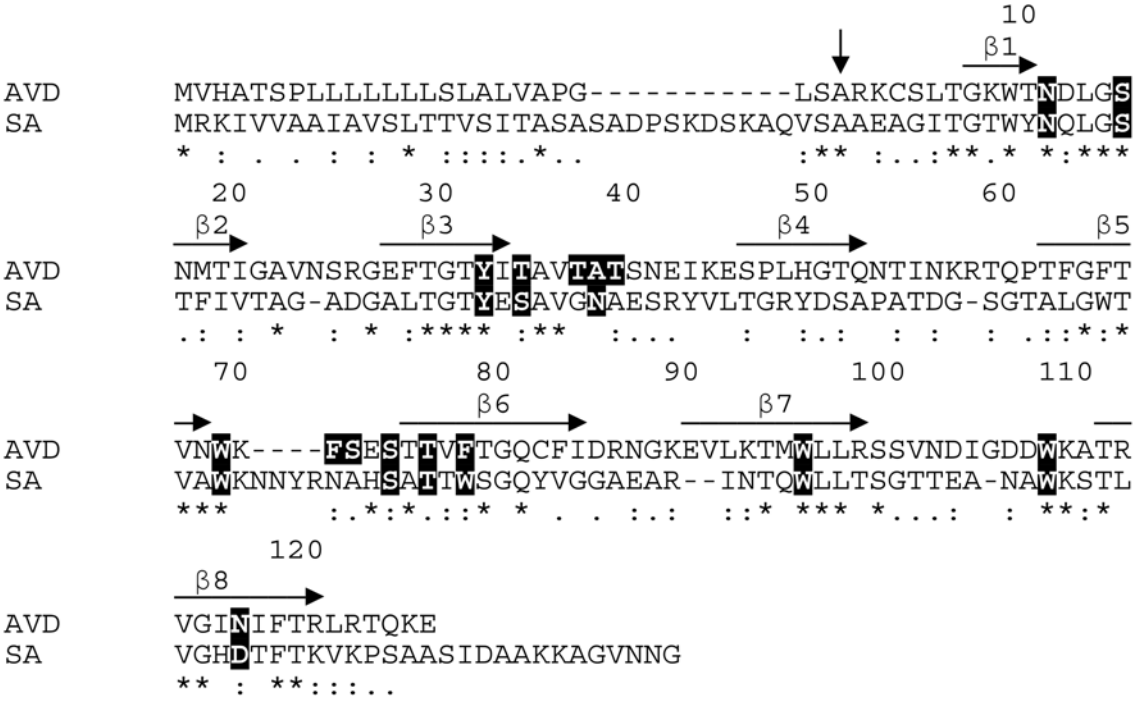

FIGURE 6 Sequence alignment of avidin (AVD) and streptavidin (SA). The secondary structure elements are indicated above the sequences according to the structure of avidin (PDB 2avi). The numbering follows the avidin sequence. Residues forming bonds with biotin in 3D-structures are shown in black shadow (PDB 2avi, PDB 1stp). Conserved residues are indicated with an asterisk (*) below the alignment, strongly similar amino acid residues are indicated with a colon (:) and weaker group similarity is indicated with a full stop (.). The cleaving site of the avidin signal peptide is indicated by a vertical arrow.

The biotin ligand binds inside the subunit of (strept)avidin, which is an eight-stranded antiparallel "up and down" beta-barrel protein. The overall shapes of the beta-barrels of these proteins are highly similar (Weber et al. 1989, Livnah et al. 1993b). The stability of the avidin and streptavidin tetramers is an interesting property, since the subunits are bound together by weak chemical interactions. Only slight changes in the amino acid composition in the subunit interface are needed to disrupt the oligomeric structure of avidin (Laitinen et al. 1999, Laitinen et al. 2001, Laitinen et al. 2003) or streptavidin (Wu & Wong 2005). The schematic presentation of the avidin tetramer is presented in Fig. 7 and the subunits are numbered according to Livnah et al. (1993b). The largest of the subunit-subunit-interfaces is the one-to-four interface, with an area of 1780 Å^2

(1.78×10^{-17} m^2). The area of the one-to-two interface is 557 Å^2 and area of the one-to-three interface only about 147 Å^2 (Laitinen 2001).

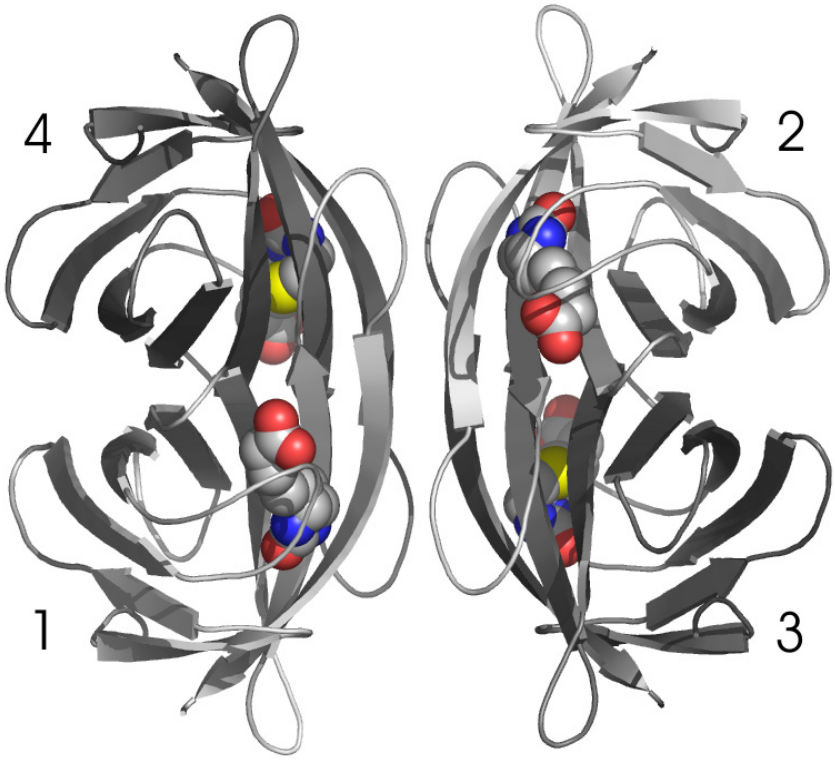

FIGURE 7 Schematic presentation of avidin tetramer numbered according to Livnah et al. (1993b). The avidin three-dimensional structure determined by X-ray crystallography was used to generate the figure (PDB 2avi). Bound biotin molecules are shown as CPK models.

The biological function of avidin is slightly controversial. Avidin protein was first detected in the chicken (Eakin et al. 1941), but is now known to be a member of the egg-white or egg-jelly proteins of avian species (Hertz & Sebrell 1942, Jones 1962, Korpela et al. 1981, Hytönen et al. 2003). The secretion of avidin from the oviduct of the chicken is induced by progesterone (O'Malley & Kohler 1967). Although avidin has been characterised as a molecule from egg-white, where it constitutes about 0.05% of the total protein, it has been detected in various chicken tissues, such as intestine, lung and pectoral muscle after systemic acute inflammatory reaction (Elo et al. 1981). The appearance of avidin can be caused by various factors, like thermal or mechanical tissue damage, *E. coli* or retrovirus infection (Elo & Korpela 1984). The avidin synthesis can be induced by bacterial lipopolysaccharide and interleukin 6 in cultured chicken myoblasts and chondrocytes (Zerega et al. 2001). Since avidin is not present protein in all vertebrates, its biological function cannot be species-universal. Avidin is thought to work as a defensive protein in the egg preventing the growth of foreign organisms by biotin depletion. The finding of an analogous protein in *Streptomyces avidinii* and *Bradyrhizobium japonicum* species supports this hypothesis (Green 1975, Nordlund et al. 2005a). Close analogues of streptavidin have also been found in *S. venezuelae*, with only a few changes in the primary structure (Bayer et al. 1995). Streptavidin was originally detected as

an antimicrobial agent preventing the growth of gram-negative bacteria (Chaiet & Wolf 1964). Zerega et al. (2001) have also suggested a role for avidin in the regulation of cell proliferation.

2.3.2 Pseudocatalytic properties of avidin

Chicken avidin has been reported to catalyse cleavage of ligand-conjugated reagents. The first detailed report concerned the HABA-acetyl ester, which was hydrolysed when bound to avidin (Vetter et al. 1994). The hydrolysis is due to presence of attacking nucleophilic residue in the vicinity of bound ligand in avidin, and Ser73 was suggested as the most likely candidate. However, the possible role of Thr38, Thr40 and Ser75 cannot be excluded on the basis of current knowledge. Interestingly, the released acetate group was found to covalently attach to avidin (Vetter et al. 1994). Furthermore, avidin was also found to be able to catalyse the hydrolysis of the biotinyl P-nitrophenyl (BNP) ester, whereas streptavidin prevents the hydrolysis of this biotin-conjugate (Huberman et al. 2001). Avidin was found to form a bond between biotin-attached BNP and Lys111 residue from the adjacent subunit, whereas this interaction was not seen in the crystal structure of the streptavidin-conjugate complex (Huberman et al. 2001). Comparison of structures of different ligand-(strept)avidin complexes (Pazy et al. 2002) has suggested that the difference in loop structures between β3 and β4 in avidin and streptavidin could be responsible for different catalytic properties. When the loop between β3 and β4 was moved to streptavidin from avidin, the resulting chimeric streptavidin was found to be almost as efficient catalyst as chicken avidin. The point mutation Leu124Arg, according to the avidin sequence, further enhanced the catalytic activity of this streptavidin mutant bringing it close to that of avidin (Pazy et al. 2003, Eisenberg-Domovich et al. 2004).

The biological role of this pseudocatalytic property is unknown. Biotin is usually attached to proteins *via* an amide bond in nature, whereas avidin is capable of catalysing the breakage of a specific ester bond between biotin and another molecule (Huberman et al. 2001). Consequently, the biological importance of this pseudocatalytic property would appear to be negligible. An example of an other type of biotin-linkage is seen in the case of biotinidase, where biotin is linked to the cysteine residue of biotinidase *via* a thioester bond (Hymes & Wolf 1999). The stability of biotinyl-biotinidase in the presence of avidin is unknown.

2.3.3 Avidin-related proteins

The avidin gene is known to coexist with several similar genes, known as avidin-related genes (*AVR*s). The initial insights into these genes came when the avidin gene was being cloned. The first cloned avidin family genes were *AVR1*, *AVR2* and *AVR3* (Keinänen et al. 1988), after which *AVR4*, *AVR5* (Keinänen et al. 1994), *AVR6* and *AVR7* (Ahlroth et al. 2000) were also cloned. The number of AVR genes seems to vary between individual chickens and even between the

cells of a single chicken, which is thought to be due to frequent recombination inside the gene family (Ahlroth 2001). According to previous studies, these genes reside close to the avidin gene in a tight 27 kb cluster in the Zq21 band in the Z chromosome of the chicken (Ahlroth et al. 2000). The chicken genome project has revealed the existence of the avidin gene together with three avidin-related genes showing similarity with *AVR2*, *AVR4/5* and *AVR6* (Niskanen et al. 2005). The sequences of the all known AVR proteins aligned with avidin are shown in Fig. 8.

The similarity between the avidin and AVR proteins is obvious since almost all residues corresponding to biotin binding in avidin are conserved in AVRs (Livnah et al. 1993b, Laitinen et al. 2002). Furthermore, the subunit interface-regions of these proteins resemble those of avidin (Laitinen et al. 2002). However, there are several differences in the primary sequences, which are reflected in the biochemical characteristics of these proteins. The isoelectric points of AVRs vary between pH 4.9 (AVR2) and pH 9.6 (AVR3). There are significantly different glycosylation site patterns in these proteins, each of which has 2–4 potential glycosylation sites per subunit, compared to the one found in avidin (Laitinen et al. 2002). An interesting feature is the orphan cysteine residue found in all AVRs excluding AVR2. This residue is located in the area corresponding to L4,5 in avidin or in the C-terminal region (only AVR4 and AVR5) (Laitinen et al. 2002).

The role of avidin-related genes is unknown. Induction of a synthesis of mRNAs of certain AVR genes has been detected in chicken tissues during inflammation reaction (Kunnas et al. 1993). Since the coding regions of the AVR genes are complete, one can assume that they are not pseudogenes. The region corresponding to the avidin signal peptide is well conserved in all AVRs (Fig. 8). Furthermore, analysis of the upstream regions of AVR genes found in the chicken genome revealed the presence of many potential promoter elements (Niskanen et al. 2005). The promoter activity of the upstream region of the *AVR4* has been shown to be dependent of the length of the upstream region used (Olukosi & Iwalokun, 2003). However, nobody has been able to detect AVR proteins in chicken tissues.

Recombinant AVR proteins have recently been produced using a baculovirus expression system (Hytönen 2001, Laitinen et al. 2002). The original signal sequences of AVRs were successfully used to secrete the proteins in insect cells. The produced proteins showed differences in glycosylation patterns and pI when compared to those of avidin. All the proteins were able to bind biotin, but only AVR4/5 showed a binding affinity comparable to that of avidin. The orphan cysteine residue was found to cause oligomerization by forming disulphide bridges between subunits from different tetramers of AVRs (Hytönen 2001). The immunological properties of AVRs differed from those of avidin. Interestingly, all the produced proteins were found to be stable in an SDS-PAGE-based thermostability assay (Bayer et al. 1996, Laitinen et al. 2002). These studies confirmed the avidin-like tetrameric basic fold of the AVRs and the functionality of the cloned genes.

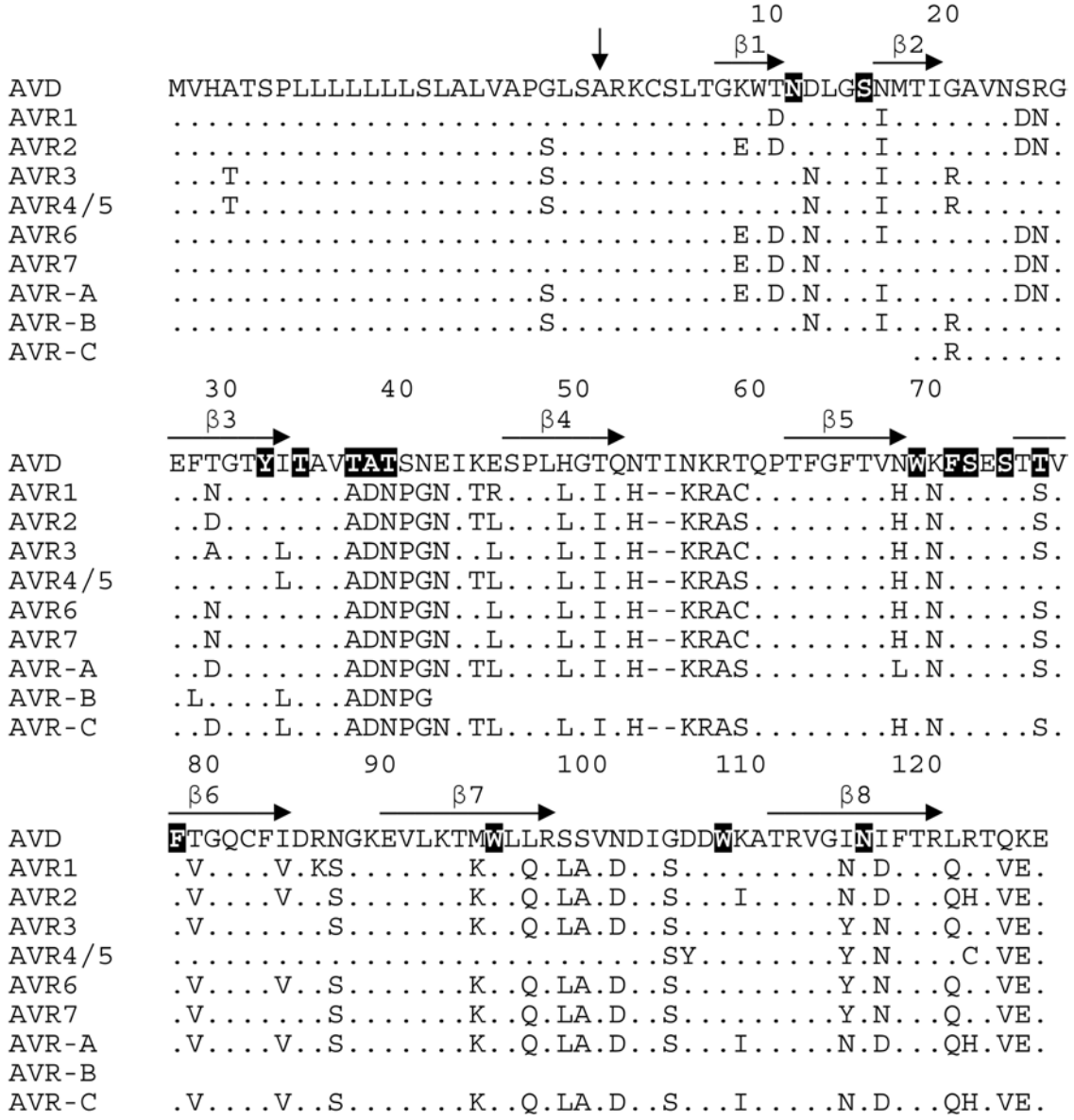

FIGURE 8 Sequence alignment of avidin (AVD) and avidin-related proteins (AVRs). The secondary structure elements of avidin are indicated above the sequences according to PDB 2avi (Livnah et al. 1993b). The numbering of sequences is according to avidin. Residues forming bonds with biotin in the avidin 3D-structure are shown in black shadow. The cleaving site of the avidin signal peptide is indicated by an arrow. AVRs 1-7 represent genes cloned previously from the chicken (Keinänen et al. 1988, Keinänen et al. 1994, Ahlroth et al. 2000). AVR4/5 is an identical product of genes *AVR4* and *AVR5*. Sequences of AVR-A, AVR-B and AVR-C were obtained from chicken genome sequencing project (Hillier et al. 2004) and these are theoretically characterised in a previous study (Niskanen et al. 2005).

2.3.4 Yolk biotin-binding proteins

There are several studies on the biotin-binding proteins present in chicken egg-yolk. White III and co-workers first characterised an egg-yolk biotin-binding protein showing a high affinity for biotin ($K_d \approx 10^{-12}$ M) and high thermal stability. Similar tetrameric quaternary structure when compared to avidin was

detected (White et al. 1976, Meslar et al. 1978). The molecular weight of this protein was estimated to be 19.5 kDa per subunit (Meslar et al. 1978). The isolated protein lacked immunologic cross-reactivity with antibodies against chicken avidin (Meslar et al. 1978). This protein has also been characterised by Murthy & Adiga 1984).

Another biotin binder from egg-yolk has also been presented, namely BBP-II (White & Whitehead 1987, Subramanian & Adiga 1995). This protein form was found to have lower thermal stability and lower affinity to biotin when compared to those of BBP-I. Furthermore, BBP-I was hypothesized to exist in a form containing all four biotin-binding subunits in the same polypeptide chain. This form was thought to be processed to another BBP form (Bush & White 1989). The three-dimensional structure of BBPs is unknown.

The analysis of the chicken genome sequencing project results yielded two genes showing similarity with the known N-terminal sequences of BBPs. These genes were found to have an exon-intron structure similar to that of the avidin gene. The avidin residues in direct contact with biotin in are highly conserved in the putative proteins of these genes whereas there is a high amount divergence in the residues located on the protein surface, according to comparative modelling. Furthermore, cDNAs corresponding to these genes have also been found to exist. Notably, one of these genes, named *BBP-A*, showed a location close to the avidin gene family (Niskanen et al. 2005).

BBPs are thought to serve as a carrier proteins, which deliver biotin to the developing chicken embryo (White & Whitehead 1987). In relation to this carrier function, BBPs bear analogy with the seed biotinylated protein (chapter 2.2.3) found in pea seeds, although the biotin-binding mechanism is different (Duval et al. 1994b). Since they have lower affinity to biotin compared to avidin, these proteins are thought to be able to release biotin when needed. No knowledge of the molecular level interactions of BBPs with other cellular components exists.

The properties of proteins known to take part in biotin binding in different organisms are summarised in Table 1.

TABLE 1 Summary of the properties of biotin-binding proteins

	Oligomeric structure	Biotin dissociation constant (K_d) (M)	Subunit size (kDa)	Organisms
Avidin	Tetramer	6×10^{-16}	15	Oviparous vertebrates
AVRs	Tetramer/oligo-tetramer	10^{-6}–10^{-14}[a]	15	Chicken[b]
Streptavidin	Tetramer	4×10^{-14}	15	*Streptomyces avidinii*
BBPs	Tetramer	1×10^{-12}[c]	15-19	Oviparous vertebrates
BPL (BirA)	Monomer (dimer)	4.5×10^{-8}[d]	60 (34)	All (*E. coli*)
Biotin transporter	?[e]	1.4×10^{-7}[f]	?[e]	All

[a]Only AVR4/5 showed significant affinity towards a 2-iminobiotin surface in this study. The obtained value gives a rough estimate of the biotin binding affinity of AVR4/5. Other dissociation constants were obtained using a biotin surface (Laitinen et al. 2002).
[b]The existence of avidin-related proteins in chicken is unknown.
[c]The form characterised by White III and co-workers (Meslar et al. 1978).
[d]BirA (Kwon et al. 2002).
[e]Information not available
[f]Biotin transporter from *E. coli* (Prakash & Eisenberg 1974).

2.4 Biotin-binding mechanism in proteins

The above-mentioned proteins all interact with biotin. The function of the proteins is reflected in their biotin-binding mode and the structure of the binding site. For example, avidin almost fully covers the bound ligand (Livnah et al. 1993b) (Fig. 7) whereas biotin has plenty of contacts with the surrounding environment when bound to BCCP (Athappilly & Hendrickson 1995) (Fig 5). Affinity for D-biotin differs significantly between proteins. Avidin is known to bind biotin most tightly and the determined dissociation constant (K_d) for the avidin-biotin complex is 6×10^{-16} M (Green 1990). The dissociation rate constant was determined with competitive displacement of radioactive biotin and the association rate constant was obtained by stopping the association reaction with excess ligand. Streptavidin also binds D-biotin extremely tightly, but around 100-fold less compared to avidin ($K_d \approx 4 \times 10^{-14}$ M) (Green 1990). BirA binds D-biotin with high affinity ($K_d \approx 4.5 \times 10^{-8}$ M). However, BirA has 1000-fold higher affinity for biotinyl-5′AMP ($K_d \approx 4.5 \times 10^{-11}$ M) as compared to biotin (Kwon et al. 2002). The BPL from the extremophile *Aquifex aeolicus* is shown to bind D-biotin with a dissociation constant of 4.4×10^{-7} M (Clarke et al. 2003).

In (strept)avidin, the biotin binds inside each subunit of the tetramer (Weber et al. 1989, Livnah et al. 1993b). The association rate of the binding reaction is rather typical for enzyme-substrate or antibody-hapten interaction but significantly slower than processes controlled by diffusion (Green 1975). Molecular dynamic simulation analysis have suggested that the binding and dissociation of biotin is accompanied by movement of water molecules through

a "channel" in the bottom of the binding site in streptavidin (Hyre et al. 2002). The biotin-binding sites consist of a number of hydrophobic and hydrophilic residues. In avidin, aromatic residues Trp70, Phe72, Phe79 and Trp97 from one monomer and Trp110 from another monomer line the biotin in the binding pocket. Streptavidin has equivalent aromatic residues, excluding Phe72, at the binding site: Trp79, Trp92 and Trp108 from one subunit and Trp120 from another. The high structural complementarity between biotin and (strept)avidin has been proved to be an important factor in the tight ligand binding (Weber et al. 1989, Livnah et al. 1993b, Pugliese et al. 1993, Dixon & Kollman 1999). The ureido oxygen of biotin is extensively hydrogen-bonded by residues Asn12, Ser16 and Tyr33 in avidin and by Asn23, Ser27 and Tyr43 in streptavidin (Fig. 9). Furthermore nitrogen-hydrogens are hydrogen-bonded *via* Ser35 and Asn118 in avidin and by Ser45 and Asp128 in streptavidin. The sulphur atom of biotin is bonded by Thr77 in avidin and by Thr90 in streptavidin. There are significant differences in the area close to the carboxylic tail of biotin. In avidin, several residues form hydrogen bonds to biotin in this region, i.e., Thr38, Ala39, Thr40, Ser73 and Ser75, whereas only two residues, Asn49 and Ser88, are hydrogen-bonded to biotin in the streptavidin holoform. This difference, together with the additional biotin-interacting Phe72 found in avidin, is thought to be responsible for the 100-fold difference in binding affinities (Green 1990, Pazy et al. 2002). The hydrogen bonds at the binding site of (strept)avidin are schematically shown in Fig. 9 (Pazy et al. 2002). The three-dimensional structure of the binding site of avidin with a bound ligand is presented in Fig. 10 (Livnah et al. 1993b).

Biotin binding to streptavidin is an enthalpically driven process (Klumb et al. 1998). From the viewpoint of binding kinetics, the extremely high affinity is due to very slow dissociation, especially in the case of avidin (Green 1975, Green 1990). To summarise, the basis of the high affinity are (1) extensive hydrophobic interactions and shape complementarity between biotin and the ligand-binding site, (2) numerous hydrogen bonds and (3) structural rearrangement of the protein in the binding process.

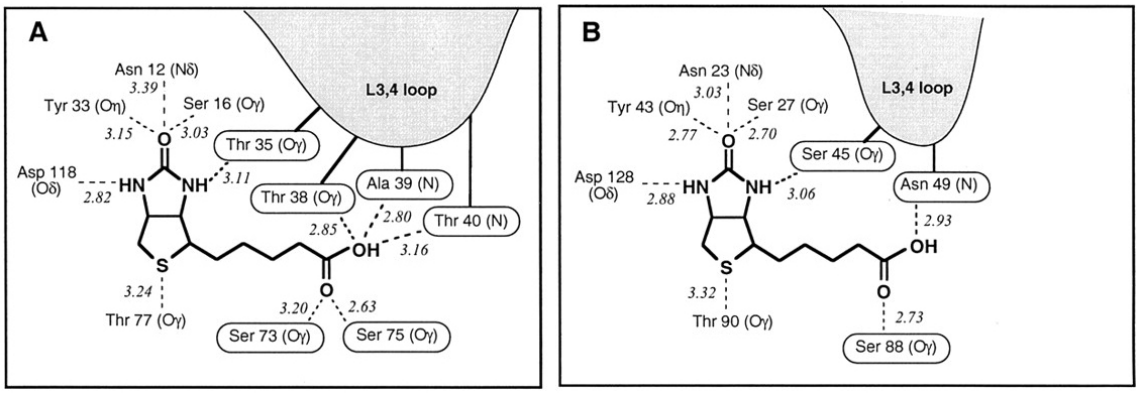

FIGURE 9 Hydrogen bonds between avidin (A) or streptavidin (B) and bound biotin. The figure is from (Pazy et al. 2002).

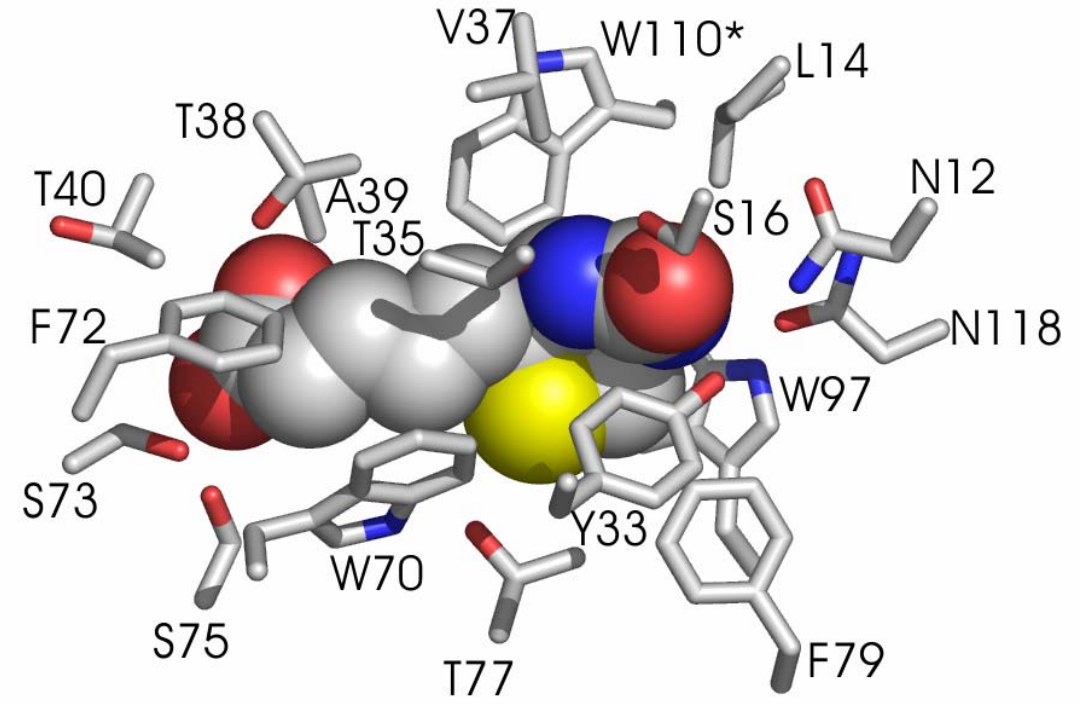

FIGURE 10 Biotin bound to avidin (PDB 2avi). Side chains of residues within 4 Å from the bound ligand are shown and labelled. The side chain of Trp110 belongs to the adjacent subunit forming part of the biotin-binding cavity. Bound biotin is presented by CPK model.

The biotin-binding site of BirA is located in the central domain of the protein (Fig. 4). Biotin makes contacts with three beta strands (β5, β8 and β9) and one helix (α5). Furthermore, loop residues 114–118 are involved in binding. Mutations Gly115Ser and Arg118Trp have been shown to decrease the binding affinity for biotin and biotinyl-5′-AMP (Kwon et al. 2002). Interestingly, loop residues 116–118, being disordered in an apoprotein, became localized upon biotin binding (Wilson et al. 1992). Biotin is extensively hydrogen-bonded at the binding site. Ureido oxygen $O^{2'}$ forms hydrogen bonds with the amide nitrogen of Arg116 as well as with the side-chain oxygen of Ser89. Ureido nitrogen $N^{3'}$ forms a hydrogen bond with carbonyl oxygen of Arg116 and biotin $N^{1'}$ is in contact with the side chains of Thr90 and Gln112 *via* hydrogen bonding. The carboxyl group in the valeryl chain of the biotin forms hydrogen bonds with Lys183 and Arg118 (Wilson et al. 1992, Weaver et al. 2001) (Fig. 11).

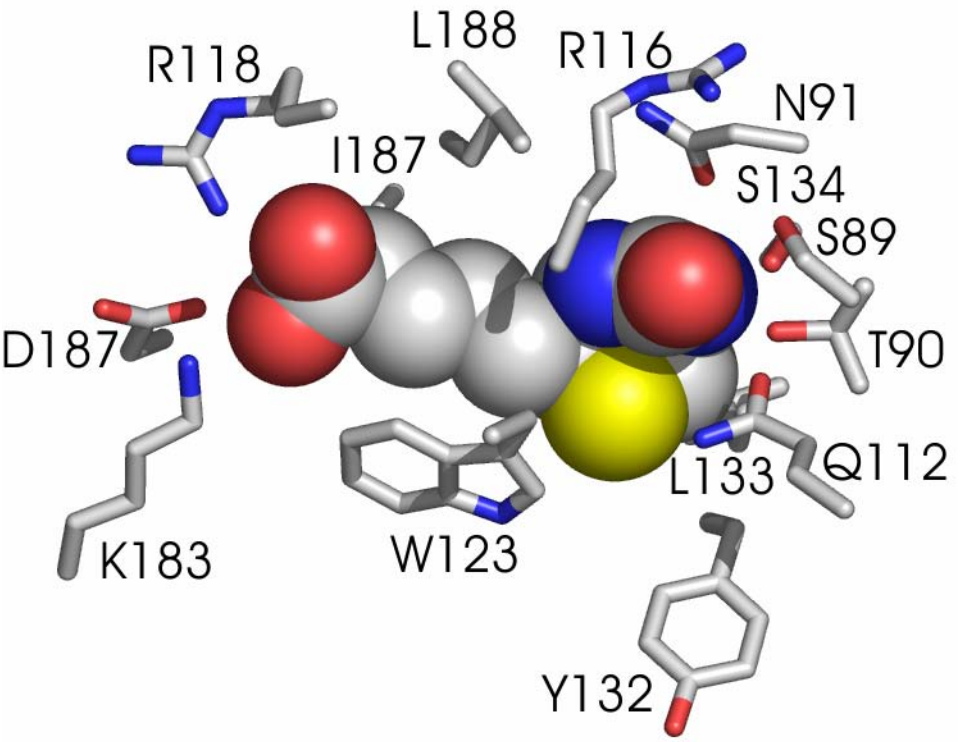

FIGURE 11 Structure of BirA complexed with biotin (PDB 1hxd) determined by Weaver et al. (2001). Side chains of residues within 4 Å from bound ligand are shown and labelled. Bound biotin is presented by CPK model.

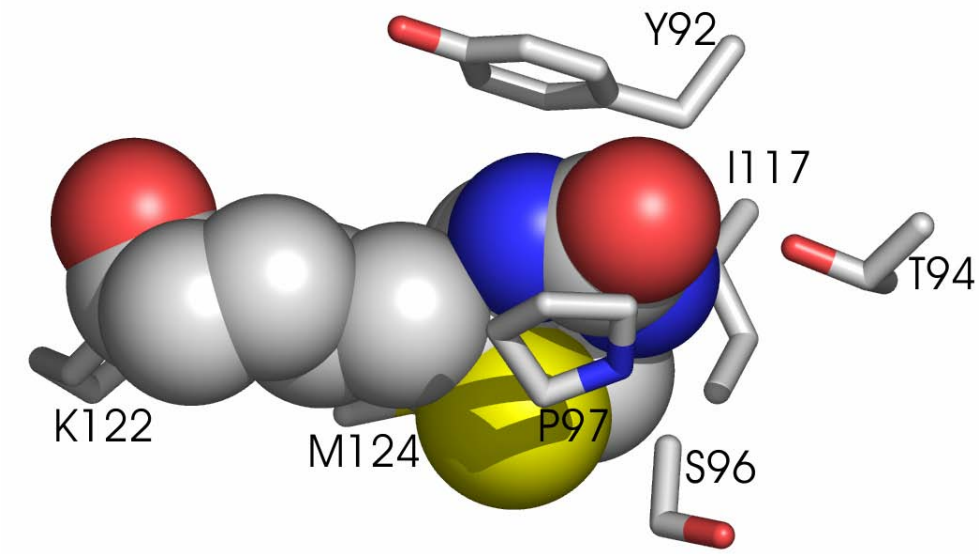

FIGURE 12 Presentation of three-dimensional structure of biotin-BCCP conjugate (PDB 1bdo) determined by X-ray crystallography (Athappilly & Hendrickson 1995). The side chains of amino acid residues within 4 Å from biotin are shown in the figure. The whole ring system of the Pro97 residue is presented for clarity. Biotin linked covalently to protein *via* Lys122 is presented by CPK model.

Although the dominant mechanism of the biotin-binding of BCCP is covalent bonding with the lysyl residue, two hydrogen-bonds, formed by Thr94 side-chain oxygen and adjacent water molecule, are present between ureido-oxygen $O^{2'}$ of biotin and BCCP, according to X-ray crystallography (Athappilly & Hendrickson 1995). Ureido $O^{2'}$ of biotin is hydrogen-bonded to the side chain of

Thr94 and ureido nitrogen to the main chain oxygen of the same residue (Athappilly & Hendrickson 1995) (Fig. 12). However, no evidence of strong interactions between these atoms has been found in NMR studies (Roberts et al. 1999). These studies have shown that the biotin group is immobilized on the surface of the protein by interactions with residues 94-101 (Roberts et al. 1999).

Albeit it is evident that these proteins are structurally unrelated, their biotin-binding mechanisms share some properties. Hydrogen bonding between biotin ureido ring atoms and these proteins is an important factor in the binding. Similarly, ureido nitrogens also form bonds with several residues. In the case of BCCP, some of the contacts found in other proteins between protein and biotin are mimicked by contacts between biocytin and adjacent water. This also reflects its function as a carboxyl carrier. The superimposition of the three-dimensional structures according to the biotin ureido ring shows that the binding patterns of these proteins are fairly similar (Fig. 13).

Biotin ureido $O^{2'}$ is a dominant atom in the bonding pattern. Hydrogen bonds are formed by three residues in avidin and streptavidin. Residues Asn12, Ser16, Tyr33 are involved in avidin (Livnah et al. 1993b) and analogous Asn23, Tyr43 and Ser45 in streptavidin (Weber et al. 1989). BirA forms contacts with biotin *via* side chains of residues Ser89 and Gln112, and the main chain oxygen of Arg116 is also involved (Wilson et al. 1992). BCCP, instead, has only a single hydrogen bond with biotin $O^{2'}$ *via* Thr94 (Athappilly & Hendrickson 1995).

Biotin $N^{1'}$ is hydrogen-bonded to the side chain oxygen of Asn118 in avidin and to the side chain oxygen of Asp128 in streptavidin (Weber et al. 1989, Livnah et al. 1993b). The nitrogen is double-hydrogen-bonded to the side chain oxygens of BirA residues Thr90 and Gln112 (Wilson et al. 1992). Analogously, biotin $N^{1'}$ forms hydrogen bond with the Thr94 side chain oxygen of BCCP (Athappilly & Hendrickson 1995). Other nitrogen, $N^{3'}$, is hydrogen-bonded with the Thr35 and Ser45 side-chain oxygen in avidin and streptavidin, respectively (Weber et al. 1989, Livnah et al. 1993b). In BirA, Arg116 main chain oxygen forms bond to this atom (Wilson et al. 1992). In the BCCP-biotin complex the $N^{3'}$ forms a hydrogen bond with the water molecule (Athappilly & Hendrickson 1995). Taken together, the ureido ring recognition of all these proteins shows rather similar extensive hydrogen bonding (Fig. 13).

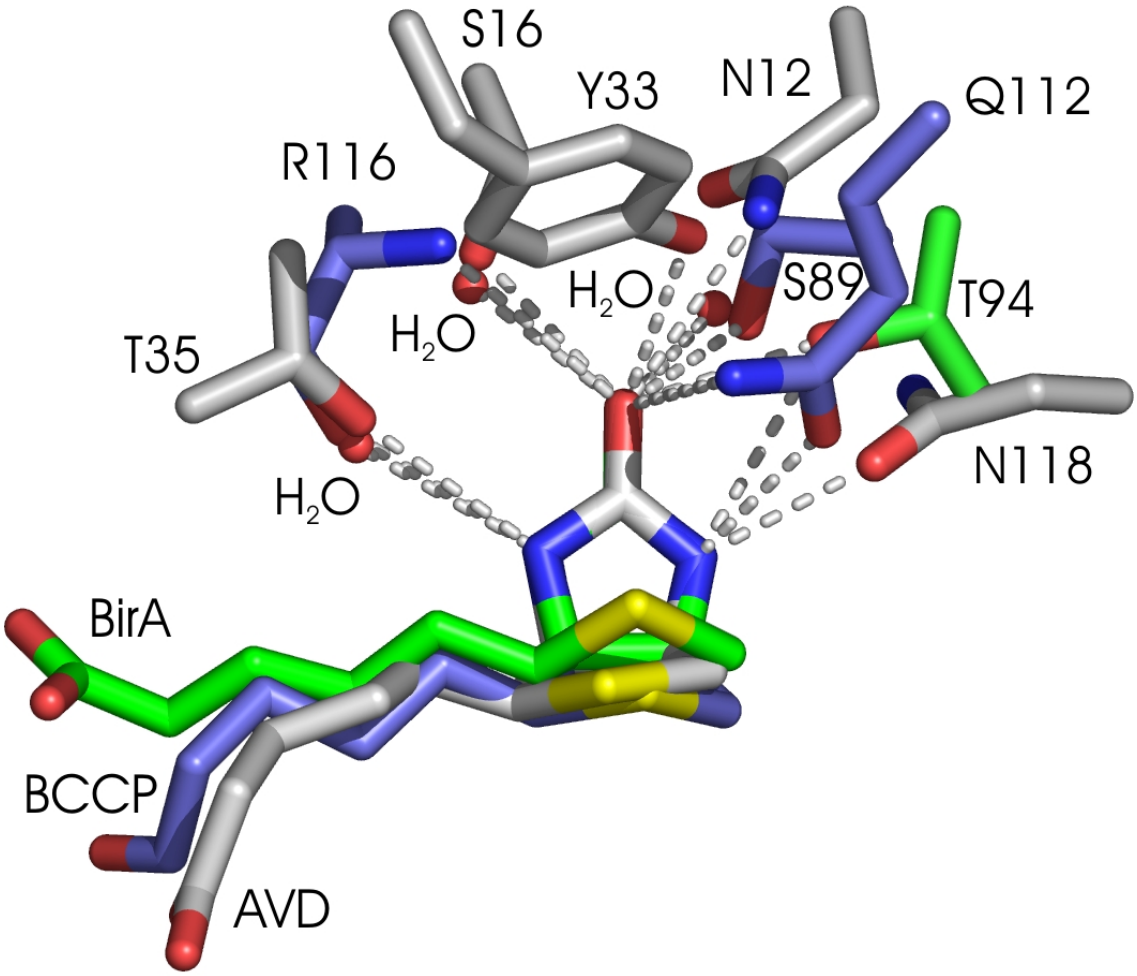

FIGURE 13 Hydrogen bonds with ureido ring atoms of biotin. The three-dimensional structures of chicken avidin (gray, PDB 2avi), BirA (green, PDB 1hxd) and BCCP (blue, PDB 1bdo) complexed with biotin are manually superimposed according to the biotin ureido ring atoms. The hydrogen bonds are shown with dashed lines. Biotins as well as side chains of contacting residues are shown as sticks. The side chain of residue R116 in BirA has been removed for clarity and only the main chain hydrogen bonds with biotin are shown. Three water molecules forming hydrogen bonds with biocytin of BCCP are displayed and labelled. The figure is partially adapted from (Athappilly & Hendrickson 1995).

The hydrogen bonding in other parts of the biotin differs markedly between these proteins. The sulphur atom of biotin is hydrogen-bonded by residues Thr77 and Thr90 in avidin and streptavidin, respectively (Weber et al. 1989, Livnah et al. 1993b). Avidin forms an extensive bonding network with carboxylic oxygens of biotin whereas streptavidin lacks most of these contacts (Fig. 11, Table 2). BirA has three residues forming hydrogen bonds with the biotin carboxyl tail and BCCP lacks hydrogen bonding in this area with biotin.

The hydrophobic characteristics of the binding site also differ between the proteins. The biotin-binding site in avidin and streptavidin is inside the protein and the overall nature of the binding site is rather hydrophobic. The hydrophobic interactions dominate the ligand binding, determined both experimentally using site-directed mutagenesis (Chilkoti et al. 1995a, Laitinen et al. 1999) and computationally by molecular dynamics simulations (Miyamoto & Kollman 1993, Dixon & Kollman 1999). The hydrophobic walls of the binding

site are formed by aromatic residues. Similar features can be seen in the binding of biotin to BirA, since one side of the biotin molecule is in contact with the hydrophobic area at the binding site (Wilson et al. 1992). The binding mode in the case of BCCP differs from that in the other biotin binders, since the bound ligand is almost totally exposed to the surrounding water (Fig. 12). However, Tyr92 makes an important exception, forming an efficient face-to-face (Athappilly & Hendrickson 1995) aromatic interaction with the tiophene ring of biotin.

TABLE 2 Comparison of the hydrogen bonding characteristics of holoforms of biotin-binding proteins determined by X-ray crystallography. The PDB accession code for protein structure used in the analysis is shown in parentheses.

	BiotinN$^{1'}$	BiotinO$^{2'}$	BiotinN$^{3'}$	BiotinS^{1}	BiotinO^{10a}	BiotinO^{10b}
Avidin (2avi)	Asn118O^{4}	Asn12O^{4}	Thr35O^{3}	Thr77O^{3}	Ser73O^{3}	Thr38O^{3}
		Ser16O^{3}			Ser75O^{3}	Ala39N
		Tyr33O^{4}				Thr40N
Streptavidin (1stp)	Asp128O^{4}	Asn23O^{4}	Ser45O^{3}	Thr90O^{3}	Ser88O^{3}	Asn49N
		Ser27O^{3}			H_2O	H_2O
		Tyr43O^{4}				
BirA (1hxd)	Thr90O^{3}	Ser89O^{3}	Arg116O	-	Arg118N	Lys183N^{6}
	Gln112O^{5}	Arg116N				Asp176O^{4}
BCCP (1bdo)	Thr94O^{3}	Thr94O^{3}	H_2O	-	-	-
	H_2O	H_2O				

As described above, hydrogen bonding is an important factor in the binding mechanism of all these biotin-binding proteins. Central binding atoms in biotin are in all cases ureido ring oxygen and nitrogens. In (strept)avidin and BirA, the hydrophobic contact between ligand and protein is extensive. If one compares the apo- and holoforms of these proteins, certain changes in the biotin-binding site can be detected. First, solvent molecules forming bonds with binding-site residues are replaced by the ligand molecule. In the case of avidin and streptavidin, five water molecules are replaced by biotin (Weber et al. 1989, Livnah et al. 1993b). Three water molecules occupy the binding site of biotin in the BirA apoform (Wilson et al. 1992). Although the overall structure of the apo- and holoforms of (strept)avidin are similar, there are slight structural changes in the binding site upon ligand binding, especially in the case of avidin. Williams et al. (2003) have shown that the structure of streptavidin becomes better packed after the binding of biotin. The loop between β3 and β4 is unstructured in the apoform of avidin but adopts a fixed conformation when biotin is bound (Livnah et al. 1993b). The corresponding loop of streptavidin is shorter and has different sequence characteristics, and it does not show similar behaviour (Weber et al. 1989). This difference is also reflected to the binding properties of these proteins, since avidin binds conjugated biotin with significantly lower affinity when compared to that of free biotin. Pazy et al. showed that the biotinyl P-nitrophenyl ester is efficiently transferred from avidin to streptavidin but not *vice versa* (Pazy et al. 2002). Although avidin binds free biotin with higher affinity as compared to streptavidin (Green 1990),

streptavidin is able to deprive the biotin-conjugate from avidin (Pazy et al. 2002). The molecular reason for this might lie in the differences in the structures of the loops between β3 and β4 (L3,4) in avidin and streptavidin. In biotin-bound form, L3,4 of avidin forms extensive contacts with biotin and almost fully covers the ligand (Livnah et al. 1993b). In contrast, L3,4 of avidin seems to partially block the binding of biotin-conjugate to the binding site and all the hydrogen bonds between L3,4 and the ligand are thus lost (Pazy et al. 2002). L3,4 of streptavidin is shorter and does not form such extensive bonding with free biotin (Fig. 9). It also seems to be able to form two hydrogen bonds with biotin-conjugate as it does with free biotin (Pazy et al. 2002).

In the case of BirA, a similar loop arrangement is seen due to the binding event. Residues 116–118, being part of the loop between β4 and β5, are disordered in the apoprotein and became visible in the holoform (Wilson et al. 1992).

2.5 Use of biotin in the life sciences

The (strept)avidin-biotin complex is stable and the affinity between these molecules is one of the highest found among non-covalent interactions in nature. Furthermore, the binding is specific [i.e. (strept)avidin does not bind other molecules with very high affinity]. These properties have made this system a very inviting scaffold on which to develop applications in the life sciences. The number of existing methods is huge, and they are roughly characterised below under three headings.

2.5.1 Biotin as a capture molecule

As mentioned, the complex formed by (strept)avidin and biotin is very stable. This property is employed in applications where biotin is covalently linked to different types of matrices, and the other molecule is captured for this matrix *via* (strept)avidin. In these systems, avidin could act as a fusion partner of other molecule (Preissler et al. 2003), displayed on the surface of the virus (Räty et al. 2004) or chemically attached to the target (Rosenberg et al. 1987). The avidin component could then be used for isolating, measuring or localisating the fusion complex. For example, liposomes have been modified by covalently attaching streptavidin to the liposome surface. Biotinylated nerve growth factor was then attached to the modified liposome surface in order to deliver liposome to the neuronal cells (Rosenberg et al. 1987).

The tetrameric structure of (strept)avidin makes it possible to use the biotin-protein pair as a signal amplifier. If a signalling molecule is coupled to biotin, up to four labels can be captured to immobilised (strept)avidin. For example, by mixing double-biotinylated label with avidin, one can obtain oligomeric structures containing several label molecules together (Crich et al. 2005). The biotin-binding capacity of a (strept)avidin surface can be even

increased by oligomerising the protein by chemical cross-linking (Välimaa et al. 2003).

Similarly, systems in which BCCP is used as a fusion partner have been developed. Fusion proteins of BCCP with several proteins are generated and BCCP biotinylated *in vivo* is then used to capture the fusion partner (Germino et al. 1993, Wang et al. 1996a, Wang et al. 1996b). Similarly, 15-residue AviTag (Avidity, Denver, CO, U.S.A.) (Schatz 1993) has been used to produce biotinylated target proteins for selection experiments carried out with phage libraries (Scholle et al. 2004). Recently, Parrott et al. were able to generate metabolically biotinylated adenovirus by genetically attaching 70-residue biotin acceptor peptide (BAP) to the C-terminus of the fiber capsid protein. The biotinylation was carried out by coexpressing *E. coli* BirA, since mammalian cells did not biotinylate this BAP (Parrott et al. 2003). The biotinylation was efficient even when 14-residue synthetic BAP was used (Schatz 1993), but the resultant virus was not active. Another biotinylation signal used is the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase. Both *E. coli* and *Saccharomyces cerevisiae* are capable to metabolically biotinylate fusion proteins containing 70- or 123-residue BAP from this domain (Cronan 1990). Recently, adenoviruses metabolically biotinylated in mammalian cells were generated by using 70-residue BAP from *P. shermanii* (Campos et al. 2004).

2.5.2 Biotin as a carrier molecule

Being a small, water-soluble molecule, biotin is easily attached to various molecules without causing changes in the function of the target molecule. Usually, the valeryl acid tail oxygen is used for coupling. The possibilities of chemical coupling methods are thoroughly reviewed by (Wilchek & Bayer) (1988). Various biotin-conjugated molecules are commercially available, for example biotinylated fluorophores. In order to use biotin as a carrier of radionuclide, radiometal chelating agents are coupled to it (Wilbur et al. 2002, Sabatino et al. 2003). These molecules are used in pretargeting cancer therapy (Paganelli et al. 1991, Paganelli et al. 1997, Paganelli et al. 1999).

Peptide nucleic acid (PNA) is one promising example of a molecular tool utilised for various purposes (Ray & Norden 2000). In biotinylated PNA, the biotin moiety works as a carrier molecule to deliver PNA to the target structure (Coester et al. 2000, Langer et al. 2000). In another example of biotinylated drugs, a tumour necrosis factor has been biotinylated and targeted to tumours using biotinylated antibody and avidin (Gasparri et al. 1999).

Paramagnetic Gd chelates are powerful reagents used in magnetic resonance imaging (MRI) (Caravan et al. 1999). Crich et al. have developed biotinylated chelates of Gd^{3+}, which could in turn be used to develop new methods for tumour imaging by MRI (Crich et al. 2005).

2.5.3 Biotin as a building block in nanotechnology

One aim of nanotechnology is to develop self-assembling structures. In this respect, (strept)avidin-biotin offers a valuable pair of molecules with very tight and specific binding properties. This possibility is used to generate oriented streptavidin-lipid layers (Blankenburg et al. 1989). These layers were also used to attach another biotinylated molecule to the formed structure (Darst et al. 1991). Carbon nanotubes could be selectively modified by avidin to obtain functional building blocks and to assemble these molecules between electrodes (Banerjee et al. 2003). Biotin-streptavidin is also used to generate a molecular valve by covalently attaching biotin to a nanotube (Hinds et al. 2004). In another study, biotinylated antibody was employed to modify carbon nanotubes to form transistors (Keren et al. 2003). Recently, Ramanathan et al. (2005) reported on functionalisation of nanowire by avidin. This system was successfully used to sense biotinylated DNA by measuring the resistance of the wire.

Avidin and 2-iminobiotin labelled poly(ethyleneimine) were previously used to generate layer-by-layer assembly (Inoue et al. 2005). The formed assembly was stable at pH 8–12 but decomposed at pH 5–6. They also found it possible to disintegrate the structure by adding competing ligands, for example D-biotin. Furthermore, the decomposition rate of the structure was varied by using different types of avidin-binding molecules.

Two recent studies have utilised (strept)avidin-biotin as a self-assembling connector between nanostructures. Caswell and coauthors (2003) were able to assemble biotin-functionalised gold nanorods using streptavidin in end-to-end orientation. Avidin was utilised in another study, where biotin-functionalised TiO_2 nanorods were assembled end-to-end (Dimitrijevic et al. 2005). Furthermore, Dimitrijevic et al. were able to specifically oxidise Tyr33 of avidin using photoexitation of the TiO_2.

2.6 Biotin analogs

2.6.1 Derivatives of biotin

Various biotin derivatives are known to bind avidin (for a thorough description see Green 1975). Some of them have been used in applications. If the ureido-oxygen of biotin is replaced with the amino group (2-iminobiotin), the resultant molecule shows pH-dependent binding to (strept)avidin, due to repulsion caused by protonation of the amino group at low pH. The dissociation constant between avidin and 2-iminobiotin has been determined to be 3.5×10^{-11} M at high pH (Green 1975); at low pH the affinity is significantly lower ($K_d < 10^{-3}$ M) (Fudem-Goldin & Orr 1990). This is used in the chromatographic affinity purification of (strept)avidin (Heney & Orr 1981). The 3D-structure of the complex of streptavidin with 2-iminobiotin has been resolved in both neutral (pH 7.3) (Katz 1997) and acidic environments (pH 2.0, pH 2.6, pH 3.25, pH 4.0).

The analysis of these complexes showed that the hydrogen bond between Asn23 and biotin is lost at low pH (Katz 1997). Another potential molecule is desthiobiotin, which lacks the tiophene ring of the biotin bicyclic ring system. This molecule has about 1000-fold lower affinity to avidin ($K_d \approx 5 \times 10^{-13}$ M, pH 7) when compared to biotin (Green 1975). Desthiobiotin is thought to be a useful molecule for efficient and reversible probing of (strept)avidin (Hirsch et al. 2002). An interesting method for specifically incorporating desthiobiotin into synthesized proteins by means of BirA has recently been published (Wu & Wong 2004).

Since the biotin carboxylic tail is easy to couple to various molecules, there are an enormous number of functionalised biotins available. Some examples can be given. Photobiotin is a biotin derivative, which can be linked to amino groups by photoactivation (Forster et al. 1985). This molecule is used for labelling DNA and RNA (McInnes et al. 1990 and references therein). Furthermore, photobiotin is used for covalent immobilization of macromolecules at defined locations on polystyrene or nitrocellulose surfaces (Hengsakul & Cass 1996). Biotin could be linked to another molecule *via* a disulphide linker, which can then be cleaved, if desired, by mild reduction (Ahmed et al. 1992). Analogously, linkers which can be cleaved by UV-illumination have also been created (Thiele & Fahrenholz 1994, Olejnik et al. 1995). The reagent published by Olejnik et al. was efficiently (> 99%) cleaved by five-minute illumination by UV light ($\lambda > 300$ nm) and the released molecule did not contain any atoms from the cleaved linker (Olejnik et al. 1995). Photocleavable biotin was recently applied to modify adenovirus. Biotinylation was found to inactivate the virus and exposure to 365 nm light reversed the viral activity (Pandori et al. 2002).

Common to all biotin attachments is the length of the linker, which should be enough to allow tight binding of biotin to the (strept)avidin binding pocket (Ross et al. 1986).

2.6.2 2-(4'hydroxyphenyl) azobenzoate (HABA)

A yellow dye molecule, 2-(4'hydroxyphenyl) azobenzoate (HABA), binds to avidin and streptavidin, occupying the same position as biotin. The binding of HABA to avidin is somewhat tighter ($K_d \approx 6 \times 10^{-6}$ M) when compared to that of streptavidin ($K_d \approx 1 \times 10^{-4}$ M) (Green 1990). The binding event is accompanied by a shift in the absorption spectra of the dye. The yellow colour of the free dye ($A_{max} \approx 350$ nm) changes to red ($A_{max} \approx 500$ nm) when HABA occupies the biotin-binding site in (strept)avidin. The three-dimensional structures of complexes between HABA and (strept)avidin have been solved (Weber et al. 1992, Livnah et al. 1993a). Analogously to biotin, HABA also displaces water molecules from the binding site. The carboxylic acid of HABA somewhat mimics the ureido oxygen of biotin, forming hydrogen bonds with the residues Asn12, Ser16 and Tyr33 in avidin (Asn23, Ser27 and Tyr43 in streptavidin) (Weber et al. 1992, Livnah et al. 1993a). Thr35 (Ser45) forms also a hydrogen bond with this carboxylic acid. The important difference in the binding mode is Asn118 (Asp128), which does not favourably interact with HABA. The

difference in HABA-binding affinities between avidin and streptavidin are thought to be due to interactions formed by the residues Phe72 and Ser73 in the loop between β5 and β6 of avidin (Livnah et al. 1993a). This loop is four residues longer in streptavidin (Fig. 6).

Weber et al. (1994) have successfully produced and studied HABA analogues. They were able to use a structure-based design to create analogues of HABA showing significantly higher binding affinity to streptavidin. The most tightly binding molecule was found to be 2-(3′3′-dimethyl-4′-hydroxyphenyl)azobenzoate (3′,5′-dimethyl-HABA), which showed 160-250 times higher affinity to streptavidin when compared to HABA. The three-dimensional structure of the (3′,5′-dimethyl-HABA)-streptavidin complex (PDB 1srj) revealed that the analog molecule showing higher affinity was able to displace six water molecules from the binding site compared to the five displaced by HABA (Weber et al. 1994). The sixth displaced water molecule is hydrogen-bonded to biotin in the streptavidin-biotin complex (PDB 1stp). No additional bonding was obtained due to modification of the ligand. Interestingly, the crystallographic studies showed that even in the case of the tight-binding HABA analogues, the ligand showed a high degree of flexibility in the bound state and even the rotation of the hydroxyphenyl ring of HABA and HABA derivatives was detected in the binding pocket (Weber et al. 1994).

Since the binding of HABA to avidin is easy to measure and could be reversed by the addition of biotin, HABA is used as a probe to count the number of biotin-binding sites available in modified (strept)avidins (Green 1970). Furthermore, methods to covalently attach the HABA *via* a linker to other molecules have been developed (Hofstetter et al. 2000). In this respect, HABA is thought to serve as an extension to the (strept)avidin-biotin system offering more variability and development of more complicated methods.

2.6.3 Peptide ligands

Peptide ligands are widely used tools in studies of protein interactions. Peptides, which occupy the binding site of biotin in avidin and streptavidin, are found in various studies. These offer powerful tools to label and isolate proteins since peptide sequences can be easily incorporated in proteins by methods of molecular biology and there are various reagents related to the (strept)avidin-biotin technology commercially available. When compared to chemical biotinylation, the number of labels per protein is better controlled. Furthermore, the use of peptide ligands also offers an advantage over *in vivo* biotinylation since there is no need to overexpress BPL. In contrast, peptide ligands have significantly lower affinity for (strept)avidin compared to biotin. By using synthetic resin-bound peptides, Lam et al. (1991) were able to find pentapeptide sequences occupying the biotin-binding site of streptavidin. All these peptides have the motif HPQ in their sequence. Utilising the same technique with D-amino acid residue octapeptides, novel motifs xRWFx, xKWxGx, xKxxWYPx and YxxxGYx were found to bind to the biotin-binding site of avidin or close to it, being displaceable with biotin (x is nonspecified residue). Analogously, D-

amino acid octapeptides with motif W(Y/H)xxAxxx were able to bind to streptavidin in a biotin-displaceable way (Ostergaard & Holm 1997).

Strep-tag is a nine L-amino acid peptide (AWRHPQFGG) that displays binding affinity towards streptavidin and has been used as an affinity tag for recombinant proteins (Schmidt & Skerra 1993). The peptide occupies the biotin-binding site in streptavidin and the three-dimensional structure of the complex is known (Schmidt et al. 1996). This peptide was further optimised in order to generate less environmentally sensitive Strep-tagII, of sequence Ac-WSHPQFEK (Schmidt et al. 1996). The acetylation in the N-terminus was included in order to mimic the preceding amino acid residue in the polypeptide chain. In order to improve the affinity of streptavidin to this new peptide, streptavidin was subjected to random mutagenesis and selection. Finally, streptavidin mutants with increased affinity to Strep-tagII were obtained (Voss & Skerra 1997). The first streptavidin mutant has residue changes Glu44Val, Ser45Thr and Val47Arg in polypeptide sequence while the second protein has mutations Glu44Ile, Ser45Gly and Val47Arg. The first mutant is commercially available under the product name StrepTactin. The three-dimensional structure of the mutated protein revealed slight differences in the binding mode of the peptide when compared to biotin. In the mutated protein complexes, the loop between β3 and β4 existed in "open" conformation also in the ligand bound state (Korndorfer & Skerra 2002).

In a recent study, a new streptavidin-binding peptide, termed Nano-tag, was obtained. Nano-tag$_{15}$ (DVEAWLDERVPLVET) showed tight binding to the biotin-binding site of streptavidin ($K_d \approx 4 \times 10^{-9}$ M). Since the shorter nine-residue Nano-tag$_9$ showed only moderate affinity to streptavidin ($K_d \approx 240 \times 10^{-9}$ M), it was further optimised by randomised mutagenesis and the final product (DVEAWLGAR) showed higher affinity to streptavidin ($K_d \approx 17 \times 10^{-9}$ M). The peptides thus obtained were used to successfully isolate fused proteins (Lamla & Erdmann 2004). In another study, a peptide library with high diversity was generated (Cho et al. 2000) and the streptavidin-binding 108-mer peptides were extracted from the library. Most of them (19/20) were found to contain motif HPQ (Wilson et al. 2001). One of them was subjected to further analysis and the sequence corresponding to high affinity was restricted to 38 residues with high affinity to streptavidin ($K_d \approx 2.5 \times 10^{-9}$ M). The resultant peptide (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP), named SBP-tag, was successfully used in single-step purifications of fusion proteins (Keefe et al. 2001).

Cyclic peptides constrain the conformational freedom of the binding motif. Cyclisation can be achieved by including cysteine residues in the sequence, causing disulphide bond formation. By using the phage-display method and screening with flow cytometry, Bessette et al. (2004) were able to isolate cyclic peptide sequences showing high affinity to streptavidin. These peptides bore consensus sequence ICMNVC. However, a lot of variance was seen in the third position of the consensus (Gln, Thr, aromatic residues). The

dissociation constants were around 5-10 nM for these peptides and the binding of the peptides was reversed by free biotin.

2.7 Modified avidins

Avidin and streptavidin share extremely high affinity to biotin. This property has encouraged scientists to study the binding mechanism and also to modify the binding process. Furthermore, several modifications have been made to (strept)avidin in order to create new tools from these proteins and to expand understanding of this intriguing protein-ligand pair.

The molecular details of of the (strept)avidin-biotin interaction have been studied in order to understand (1) biotin-binding thermodynamics, (2) roles of aromatic interactions in biotin binding and (3) roles of hydrogen bonds in the binding process. Modification for biotechnological purposes has been done in order to (1) modify the charge and glycosylation of the proteins, (2) modify the oligomeric properties of (strept)avidin, (3) lower the biotin-binding affinity in order to create tools for specific applications, (4) increase the stability of (strept)avidin and (5) produce chimeric forms of (strept)avidin where different functions can be linked in one quaternary unit.

2.7.1 Studies on biotin binding

Since (strept)avidin is a biologically interesting protein and an efficient biological tool, much effort has been expended on studies concerning the modification of the protein for different purposes. The first studies employed chemical modification. Positively charged avidin was chemically neutralized *via* lysine residues by formylation (Guesdon et al. 1979), acetylation (Kaplan et al. 1983) or succinylation (Finn et al. 1984). Furthermore, avidin can also be neutralised *via* arginine residues. This protein is commercially available (ExtrAvidin®, Sigma), allowing one to use lysine side chains for further modification. Furthermore, Belovo S. A (Bastogne, Belgium) offers similarly neutralised, enzymatically deglycosylated avidin (NeutraLite Avidin™).

Chemical modification has also been used to study the binding properties of avidin. Bayer and Wilchek with their coworkers used reagents specifically modify lysine (Gitlin et al. 1987), tryptophan (Gitlin et al. 1988) and tyrosine (Gitlin et al. 1990) residues in order to study their importance for the function of avidin. Later, mutagenesis and recombinant protein expression has been used in various studies in order to study and modify strept(avidin). Stayton and his group mutated tryptophans 79, 108 and 120 in streptavidin to alanine or phenylalanine and characterised the biotin-binding properties of the resulting mutated proteins (Chilkoti et al. 1995a). Mutation to alanine caused a huge drop in binding affinity in each case, whereas phenylalanine mutants Trp79Phe and Trp108Phe still showed rather high affinity to biotin. Mutation Trp120Phe caused a significant decrease in binding affinity as compared to wt. Similarly,

mutation Trp110Lys in avidin and Trp120Lys in streptavidin caused a significant decrease in binding affinity (Laitinen et al. 1999). Mutation Trp110/120Lys was done according to the protein sequence originating from the sea urchin fibropellin avidin-like domain. Recently, this fibropellin domain was shown not to bear biotin-binding activity (Itai et al. 2005).

Other studies have concentrated on the residues forming hydrogen bonds to biotin. Klumb et al. (1998) mutated residues forming hydrogen bonds to ureido $O^{2'}$ of biotin to alanine in streptavidin, and all them (Asn23Ala, Ser27Ala, Tyr43Ala) an approximately 100-fold decrease in binding affinity. Mutation Tyr43Phe weakened only slightly the ligand-protein complex and Asn23Glu caused an effect milder than Asn23Ala. We studied the effect of analogous mutation Ser16Ala in avidin, observing decrease of about 10-fold in biotin-binding affinity (Hytönen et al. 2005). Furthermore, avidin mutants Tyr33Ala, Tyr33Phe, Tyr33His and Tyr33Gln were produced and characterised in our laboratory (Marttila et al. 2003). Mutation Tyr→Ala and Tyr→Phe were observed to cause effects similar to those in streptavidin (Klumb et al. 1998). Interestingly, we observed pH-dependent binding behaviour on the part of the Tyr33His mutant (Marttila et al. 2003).

Aspartate residue 128 forms a hydrogen bond to biotin ureido $N^{1'}$ in streptavidin. Freitag et al. (1999) converted this residue to alanine obtaining a significant decrease in binding affinity. In an analogous study, Qureshi et al. (2001) found this mutant to partially disrupt the oligomeric nature of streptavidin. Hyre et al. (2000) broke the hydrogen bonding between $N^{3'}$ of biotin by mutating serine 45 to alanine. Analogously to mutation Asp128Ala in streptavidin (Freitag et al. 1999c), they found that the mutated residue plays a thermodynamically important role in the ligand-protein complex. Interestingly, Qureshi et al. (2001) have found that this mutation also destabilises the tetramer of streptavidin. We studied the analogous site in avidin by applying mutation Thr35Ala. We observed a decrease of about 100-fold in biotin-binding affinity due this modification (Hytönen et al. 2005). Finally, the importance of the hydrogen bond between biotin S^1 and threonine residue 90 in streptavidin have been studied by mutagenising the residue to alanine. The resultant protein existed as a mixture of tetrameric and monomeric forms, the tetrameric forms exhibiting high affinity to biotin (Qureshi et al. 2001). Furthermore, Qureshi et al. have also combined Ser45Ala, Thr90Ala and Asp128Ala mutations within the same protein, obtaining fully monomeric streptavidin (Qureshi et al. 2001).

One interesting study has focused on the change in ligand selectivity of streptavidin. Mutated protein, in which asparagine 23 was converted to alanine and serine 27 to aspartate, showed higher affinity to 2-iminobiotin than to biotin (Reznik et al. 1998).

In a study performed by phage display and shotgun scanning, effects arising from alanine substitutions were studied. Notably, several residues reflecting to streptavidin-biotin interaction far from the binding site residues were detected. For example, mutations Tyr96Ala, Arg103Ala, Ile104Ala and Phe130Ala had high impact for the function of streptavidin. Previously

characterised substitutions Trp108Ala and Asp128Ala were also found to be effective in blocking biotin binding (Avrantinis et al. 2002).

2.7.2 Studies on other biochemical properties

Several studies have focused on the oligomerization of (strept)avidin. The commercial affinity columns containing monomeric avidin are produced by chemically dissociating monomers from the tetramers (Immobilised Monomeric Avidin, Pierce, Rockford, IL, U.S.A.; SoftLink™ Soft Release Avidin, Promega, Madison, WI, U.S.A.). In other approaches, mutagenesis has been applied to the subunit interfaces to prevent the tetramerisation of the protein. Most studies have concentrated on the interface between subunits one and three in (strept)avidin (Fig. 7). This interface is mainly formed of three residues pointing to each other.

The largest interface between subunits one and four was mutagenised by our group (Laitinen et al. 2001). Mutated avidins, Asn54Ala and Asn54Ala+Asn69Ala double mutant, were found to be monomeric in solution and stabilised in tetrameric form in the presence of biotin. In another study, drastic mutation to the small interface between subunits one and two, Trp110Lys in avidin and Trp120Lys in streptavidin, caused the appearance of dimeric protein forms and decreased the stability of the proteins (Laitinen et al. 1999). Combination of mutations Asn54Ala and Trp110Lys produced avidin, which appeared to be monomeric in solution both in the absence and presence of biotin and to have a moderate affinity to biotin ($K_d \approx 10^{-7}$ M) (Laitinen et al. 2003).

Qureshi et al. (2001) have found that streptavidin oligomer stability can be modified by applying alanine mutations to residues Ser45, Thr90 and Asp128. Nordlund et al. (2003) used a different approach to modify the subunit association by replacing the interface residues with histidines. We observed a pH-dependent oligomerisation in the case of mutations Met96His and Val115His, since mutation Ile117His showed no change in the oligomerisation properties of avidin (Nordlund et al. 2003b). Avrantinis et al. (2002) have found wt residues to be strongly selected over alanine mutants in shotgun mutagenesis experiments in positions 125 and 127 corresponding to interface residues 115 and 117 in avidin, respectively. Most interestingly, they found that mutation Gln107Ala (corresponding to residue Met96 in avidin) was preferred at the interface between subunits one and three of streptavidin, possibly reflecting increased stability of the mutated protein compared to wt.

2.7.3 Studies on topological properties of avidin

Circularly permuted streptavidin in which the residues forming loop between β3 and β4 was removed has previously been described (Chu et al. 1998). This protein formed tetramers in solution. By using this strategy Stayton and coworkers were able to study the effects of loop deletion to biotin-binding in

streptavidin without the effect of structural perturbation resulting from conventional loop truncation.

(Strept)avidin is a homotetrameric protein. This property limits the modification of the protein such that every new modification is simultaneously subjected to every subunit. Different types of approaches have been used to address this issue. The first attempt was made by Chilkoti et al. (1995). Different types of streptavidin subunits were mixed using the denaturation-renaturation method. Although this strategy proved successful in principle, the process yielded heterogenous material (Chilkoti et al. 1995b).

Recently, a dual chain avidin (dcAvd) was created (Nordlund et al. 2004). In that study, two avidin subunits were cloned together in the same polypeptide chain. Since the distance between the C/N terminuses of different subunits in the 3D-structure of the avidin tetramer (Livnah et al. 1993b) is rather long, a simple fusion of two avidins did not allow this kind of molecular construction without an extremely long linker between subunits. Therefore, circularly permuted avidins were first created such that the original termini were genetically joined together *via* a peptide linker and the new termini were positioned so as to reside close to each other in the 3D-structure. Two differently circularly permuted avidin forms were created, and these were joined together by a three-residue linker to form DNA encoding for dcAvd. Notably, dcAvd was found to have avidin-like high biotin affinity. The stability of dcAvd was slightly lower that that of avidin. This new avidin scaffold allows one to independently modify the two biotin-binding subunits in the polypeptide chain.

In another of our studies, dual chain avidin was employed to create avidins exhibiting different biotin affinities between biotin-binding units inside the quaternary unit (Hytönen et al. 2005). The mutagenesis was targeted to biotin-binding residues Ser16, Thr35 and Tyr33 of circularly permuted avidin cp6→5 in dcAvd. Furthermore, one point mutation, Ile117Cys, was targeted to cp5→4 component of the dcAvd in order to create a disulphide bridge between the subunits.

In our most recent study, a single chain avidin (scAvd) was created by joining together two dual chain avidins (Nordlund et al. 2004) by a 12-residue peptide linker (Nordlund et al. 2005b). The resultant scAvd exhibited tight biotin conjugate binding comparable to those measured for dcAvd and avidin. The radiobiotin dissociation assay showed a slightly lower dissociation rate constant for scAvd when compared to those of avidin and dcAvd. The scAvd was found to be thermally less stable when compared to avidin but almost as stable as dcAvd.

3 OBJECTIVES OF THE STUDY

Several years ago, when molecular cloning of the avidin gene was attempted, a number of avidin-related genes were found. Analysis of these genes revealed that they might not simply be wasted DNA but able to code functional proteins. To date, nobody has been able to detect these proteins in chicken tissues, although traces of mRNA have been detected. In a previous study, expression of the cDNAs of AVRs yielded recombinant proteins showing tight biotin binding and interesting physicochemical properties. In the present study, AVR4/5 was chosen for further characterisation due to its promising properties when compared to those of avidin and streptavidin.

The aims of the study were:

1. To produce active avidin in *E. coli* employing a signal peptide from a bacterial protein.
2. To study the thermal stability of AVR4/5 and the role of N43 and C122 in thermal stability.
3. To characterise the biotin-binding properties of AVR4/5.
4. To produce a chimeric form of avidin and AVR4/5.
5. To determine the 3D structure of AVR4/5 in order to gain a detailed understanding of its high thermal stability and differences in biotin binding compared to avidin.

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods are described in details in the original publications I-IV.

4.1 Construction of the expression vector for bacterial avidin

Avidin cDNA from plasmid pGem+Av (Airenne et al. 1994) was used as a template in this work. The cDNA was extended by using the stepwise elongation PCR method (SES-PCR) (Majumder 1992) to include the *Bordetella avium* OmpA secretion signal (Gentry-Weeks et al. 1992) peptide encoding region and flanking attL-recombination sites (Gateway, Invitrogen) (Hartley et al. 2000) in the construct (I).

The DNA products obtained from SES-PCR were cloned into pBVboostFG (Laitinen et al. 2005) by the Gateway LR-cloning reaction (Invitrogen). The obtained construct was confirmed by sequencing (I).

4.2 Mutagenesis of avidin and avidin-related protein 4/5

The avidin cDNA was obtained from plasmid pGem+Av (Airenne et al. 1994) (I, IV). *AVR* cDNAs (Keinänen et al. 1988, Keinänen et al. 1994, Ahlroth et al. 2000) were cloned to pFASTBAC1 plasmid by Dr. Mervi Ahlroth, Eevaleena Porkka and Sanna Heino. These constructs were mutagenised by using either the QuikChange (Stratagene, La Jolla, CA, USA) or megaprimer method (Sarkar & Sommer 1990) (II). Expression construct for ChiAVD was created by producing two megaprimers from avidin cDNA and using these megaprimers in PCR reaction with AVR4/5 cDNA as a template. The product was cloned to

pFASTBAC1 plasmid and further mutagenised by the QuikChange method (IV). The constructs thus obtained were confirmed by DNA sequencing.

4.3 Protein expression and purification

Protein expression in *E. coli* (I) was done by using cell line BL21(AI) (Invitrogen). The T7 polymerase gene is under an extremely tight arabinose-inducible promoter in this strain, and the expression of the T7 polymerase was further repressed by glucose. The electrocompetent cells were transformed by electroporation and allowed to grow in LB plates supplemented with gentamycin, tetracycline and 0.1 % glucose overnight at 37 °C. The resultant colonies were inoculated to 5–50 ml LB medium with both antibiotics and 0.1 % glucose and grown overnight at 25-30 °C. The bacterial suspension was diluted to the final growth volume and allowed to grow at 25-30 °C until A_{595} reached 0.3-0.6 and induced by adding 0.2 % (*w/v*) L-arabinose. The incubation of the suspended cells was continued overnight (12-15 h) at 200 rpm. Bacterial cells were collected by centrifugation and suspended in hypertonic buffer supplemented with lysozyme. After sonication, the extract was diluted to HilloI buffer and centrifuged (15000 g, 15 min). Soluble proteins were isolated and purified from the supernatant by using 2-iminobiotin affinity chromatography essentially as described previously (Airenne et al. 1997) (I).

The pFASTBAC1-cloned expression constructs were used to create recombinant baculovirus genomes by using homologous recombination in *E. coli* DH10BAC cells according to the Bac-to-Bac expression system instructions. The recombinant viral genomes were then used to transform *Spodoptera frugiperda* Sf9 insect cells. Recombinant viruses were collected from the growth medium after three days incubation (II, IV).

Protein expression in insect cells was performed using *Spodoptera frugiperda* Sf9 cells grown in SF 900IISFM (Gibco BRL), HyQ SFX (HyClone, Logan, UT) or Biowhittaker INSECT-XPRESS medium (II-IV). Before infection with recombinant baculoviruses, the cells were moved into a biotin-free medium. The infections were continued for 72 hours and cells were collected by centrifugation and stored at –20 ^{o}C until used for the analyses. Protein isolation was performed essentially as described previously (Airenne et al. 1997). Cells were suspended in a lysis buffer and destroyed by sonication. Soluble protein was obtained from the supernatant after centrifugation. Proteins were isolated and purified from the supernatant by using 2-iminobiotin affinity chromatography. If necessary, the proteins were dialysed in appropriate buffer (II, IV).

4.4 Characterisation of the proteins

4.4.1 Ligand-binding analysis

Optical biosensor. As previously in numerous studies in our laboratory (Marttila et al. 1998, Laitinen et al. 1999, Marttila et al. 2000, Laitinen et al. 2001, Laitinen et al. 2002, Hytönen et al. 2003, Laitinen et al. 2003, Nordlund et al. 2003b, Nordlund et al. 2003a, Nordlund et al. 2004), an IAsys optical biosensor was used to measure the biotin-binding kinetics of the avidin forms (I, II, IV). A carboxymethyldextrane cuvette was functionalised by coupling 2-iminobiotin covalently to the surface. By using the 2-iminobiotin surface as a probe, apparent K_d-value as well as association (k_{ass}) and dissociation rate constant (k_{diss}) were determined for the protein under study. Borate buffer (50 mM, pH 9.5) containing 1 M NaCl was used in the measurements (I, II, IV). Commercial biotin-functionalised aminosilane cuvettes were also used to measure the relative tightness of the biotin-protein bond by one-hour competitive incubation in the presence of free D-biotin (Sigma) (II). The biotin surface measurements were performed in PBS containing 1 M NaCl.

Competitive avidin dissociation from the 2-iminobiotin surface was performed by allowing the protein under study (2–5 µM) to bind at equilibrium in 50 mM borate buffer containing 1 M NaCl (pH 9.5) after which the cuvette was washed two times with buffer. Biotin solution was then added to a final concentration of 0.1 mM and the dissociation of the protein from 2-iminobiotin surface was followed for 500 seconds. The data was analysed by fitting single-phase dissociation curve to the data using the FastFit program.

Fluorescence spectroscopy. In order to study the biotin-binding properties in solution, the biotinylated fluorescent probe ArcDia BF560™ was used (I, IV). In principle, the developed assay is based on the fluorescence quenching of the probe due to binding to the avidin protein. The dissociation of the complex thus formed is then visualised by applying 100-fold molar excess of free biotin to the sample. The assay was performed at room temperature (I, VI) and also at elevated temperature (50 °C) by using a circulating water bath (IV). The sample was continuously mixed by a magnetic stirrer built into the instrument. The data were analysed by fitting the single-phase dissociation model to the data, as described previously (Hamblett et al. 2002) (Eq. 5).

$$-k_{diss}t = \ln(B/B_0) \qquad \text{Eq. 5}$$

In the equation, B is the measured amount of the complex at a certain time point and B_0 is the determined initial binding. The maximum binding was determined by measuring the difference in fluorescence of the free fluorescent biotin and the equilibrated (quenched) fluorescence signal 500 seconds after addition of the equimolar avidin sample. An avidin sample saturated with free biotin was used as a control in this assay.

Calorimetry. The thermodynamics of the biotin binding was studied by analysing the binding reaction and the complex thus formed by isothermal calorimetry (ITC) and differential scanning calorimetry (DSC). The enthalpy of the binding reaction was measured by ITC at 25 °C and 37 °C. The increase in the melting temperature (T_m) of the protein due to biotin binding was measured by DSC. In order to determine the ΔC_{pL}, the ΔT_m was also measured in pHs 4.0 and 7.0. Furthermore, the ΔH of the protein unfolding in the absence of the ligand was determined from the DSC data. The measured values were used to calculate the binding affinity in (T_m) according to Brandts & Lin (1990) (Eq. 6).

$$K_b(T_m) = \{\exp[-\Delta H(T_0)/R(1/T_m - 1/T_0) + \Delta C_p/R(\ln T_m/T_0 + T_0/T_m - 1)] - 1\}/[L]T_m \quad \text{Eq. 6}$$

Affinity at room temperature was then calculated according to Eq. 7 (Brandts & Lin 1990).

$$K_b(T) = K_b(T_m)\exp[-\Delta H_L(T)/R(1/T - 1/T_m) + \Delta C_{pL}/R(\ln T/T_m + 1 - T/T_m)] \quad \text{Eq. 7}$$

Radioactive biotin. The biotin binding kinetics of various proteins were also analysed by radiobiotin dissociation assay with [^{3}H]biotin (Amersham Biosciences), as previously described (Klumb et al. 1998). In this assay, the radioactive biotin is first allowed to bind to avidin and its dissociation from protein is observed when 1000-fold molar excess of free biotin is applied to the sample. The data were analysed using the global fit method according to Hyre et al. (2000). In this method, all the data measured at the various temperatures are used to construct a model of the temperature-dependence of the dissociation based on the Eyring equation for the dissociation rate constant (Eq. 8).

$$k_{diss}(T) = k_B T/h \exp(T\Delta S^{\ddagger} \Delta H^{\ddagger}/RT) \quad \text{Eq. 8}$$

In Eq. 8, k_B is the Boltzmann's constant, h is the Planck's constant and the R is the universal gas constant. Values for the activation entropy change term $\Delta S^{\ddagger}$ and for the activation entalphy change term $\Delta H^{\ddagger}$ are obtained from the fitted data.

4.4.2 Stability analysis

Calorimetry. The thermodynamics of the denaturation of the proteins was analysed by means of differential scanning calorimetry (DSC), as previously described (Gonzalez et al. 1999, Nordlund et al. 2003a). A Calorimetry Sciences Corporation (CSC) Nano II differential scanning calorimeter was used in the measurements. The transition midpoint of heat denaturation (T_m) of the various avidins was measured. The T_m was also determined in the presence of three-fold excess of biotin in the sample. Change in the enthalpy of the unfolding (ΔH) was also calculated from the obtained data. A 50 mM Na-PO_4 buffer with 100 mM NaCl (pH 7.0) was used in the measurements.

SDS-PAGE assay. The stability of the oligomeric assembly in the presence of detergent was studied by an SDS-PAGE-based method (Bayer et al. 1996). Prior to analysis, proteins were acetylated *in vitro* by a N-hydroxy succinimide acetic acid ester. Samples containing biotin were also prepared and analysed. Each protein was exposed to various temperatures in the presence of SDS and 2-mercaptoethanol for 20 minutes. After heat treatment, the oligomeric state was assessed by analysing the sample by SDS-PAGE (Laemmli 1970) followed by Coomassie staining. The transition midpoint of oligomeric disassembly (T_r) was determined from the data in order to resemble a temperature where half of the protein sample was in the monomeric state.

Microparticle analysis. In order to determine the heat durability of the immobilised avidin samples, avidin-coated plastic microparticles were heated at 95 °C for 30 min. After the heat treatment, the ability of the particles to bind fluorescent biotin conjugate ArcDia™ BF523 was analysed by measuring the sample with an ArcDia TPX PlateReader instrument (Soini et al. 2000) using two-photon excitation (Callis 1997). These measurements were also carried out by preincubating the sample with the fluorescent biotin conjugate before heat treatment. Negative control was prepared by saturating the avidin-coated microparticle with excess biotin.

Microplate assay. Microplate assay was used to measure the residual biotin-binding capacity of avidin sample as a function of time at 99.9 °C similarly as described in Nordlund et al. (2003a). The protein was diluted to 5 µg/ml in 50 mM Na-PO_4 buffer with 100 mM NaCl (pH 7.0) and treated with heat for the defined time in PCR apparatus with heated lid (102 °C). The biotin-binding activity of the protein was then analysed by coating a Nunc Maxisorp plate with the protein and probing the wells with biotinylated alkaline phosphatase.

4.4.3 Quaternary structure analysis

The oligomeric state of the proteins was analysed by FPLC gel filtration analysis. A Shimadzu chromatography instrument (LC-10AT_{VP}) equipped with Amersham Superdex HR10-30 column was used in the analyses. The liquid phase was 50 mM Na-PO_4 buffer with 650 mM NaCl (pH 7.0) or 50 mM Na-carbonate buffer with 150 mM NaCl (pH 11.0). The elution of the sample from the column was detected by an absorbance Diode array detector (SPD-M10A_{VP}), and by measuring the intrinsic fluorescence of the proteins at 350 nm with a fluorescence detector (RF-10A_{XL}). An excitation wavelength of 280 nm was used.

4.4.4 Structural analysis by X-ray crystallography

AVR4/5(C122S) produced in insect cells was crystallised by the vapor diffusion hanging drop method at 20 °C. Equal volumes of the protein (7.5 mg/ml) and the reservoir solution (2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6, with or without 10 % glycerol) were added to a 4-µl drop. The crystals thus obtained were soaked in a cryoprotectant solution containing 2.0 M ammonium

sulfate, 0.1 M sodium acetate pH 4.6, and 25 % glycerol. Diffraction data were collected at 100 K using an Oxford Cryosystem Cryostream cooling device from a single crystal on an ADSC Quantum 4R CCD detector with an oscillation range of 0.5° at beam line ID14-2 (λ = 0.933 Å) in The European Synchrotron Radiation Facility (ESRF). The crystal belonged to the tetragonal space group $P4_12_12$ with cell parameters *a* = 80.96 Å, *c* = 140.70 Å. Data were integrated, reduced and scaled using the HKL suite (Otwinowski & Minor 1997).

AVR4/5(C122S) expressed in *E. coli* was crystallized in the apo and biotin-complexed forms *via* the vapor diffusion hanging drop method in a reservoir solution containing 1.7–2.3 M sodium formate, 0.1 M sodium acetate (pH 4.2). Biotin was complexed to protein prior to crystallization by adding a saturated solution of biotin. The cryoprotectant solution contained 2.2 M sodium formate, 0.1 M sodium acetate buffer, pH 4.6, and 25 % – 30 % glycerol. The diffraction data of both the native and biotin-complexed crystals were collected as described above at beam line ID14-4 at ESRF (λ = 0.9393 Å). Data were integrated and scaled using the HKL suite (Otwinowski & Minor 1997). The crystals belonged to the $P4_12_12$ space group with cell parameters of *a* = 78.04 Å, *c* = 110.80 Å and *a* = 78.04 Å, *c* = 110.03 Å for the native and biotin complex, respectively, with two monomers in the asymmetric unit.

4.5 Computer programs

Most of the work with DNA sequences was done using DNAMAN 4.11 (Lynnon BioSoft, Quebec, Canada). Visualisation of three dimensional protein structures and molecular modelling were done by BODIL (Lehtonen et al. 2004). Pictures from structure coordinates were generated using PyMOL (DeLano 2005) and further edited using Corel DRAW Graphics Suite. Primary sequence analysis of the proteins were done by ProtParam (Gill & von Hippel 1989). Signal peptide characteristics were studied using SignalP (Bendtsen et al. 2004). Multiple sequence alignments were done by CLUSTALW (Thompson et al. 1994). ISIS/Draw 2.4 (MDL Information Systems, Inc., San Leandro, CA, U.S.A.) was used to produce Fig. 2.

5 REVIEW OF THE RESULTS

5.1 Production of avidin in *E. coli* (I)

Chicken avidin has been proved to be valuable tool in the life sciences. Although various expression systems for the production of avidin have been published, none of them offer as much feasibility as the simplest system used in laboratories, i. e., bacteria. The aim of this study was to develop a method of secreting the avidin protein in *E. coli*. Furthermore, recombinatorial cloning cassettes (Hartley et al. 2000) were included in the construct to develop a tool for library screening purposes.

The chosen OmpA bacterial secretion signal (Gentry-Weeks et al. 1992) was included in the avidin cDNA sequence (Gope et al. 1987) by using stepwise elongation PCR (Majumder 1992) (I, Fig. 1). The DNA product thus obtained was then cloned to the pBVboostFG multipurpose vector (Laitinen et al. 2005) and validated by sequencing. In addition, a construct where sequence coding for the natural signal peptide of the avidin (Gope et al. 1987) was created using identical methods.

The use of the bacterial signal peptide increased significantly the yield of active avidin obtained by bacterial cultivation (about 10 mg/l) when compared to the construct exploiting the native signal peptide (< 1 mg/l). The signal peptide was found to be cleaved by immunoblot analysis although a significant amount of intact polypeptide was also detected (I, Fig. 2). The yield after one-step 2-iminobiotin affinity chromatography purification (Airenne et al. 1997) ranged from 5 to 10 mg pure protein per one litre of bacterial culture medium (I, Table 3).

The obtained protein was characterised by using commercial chicken avidin as a control protein (I, Table 2). The protein obtained by bacterial expression was found to have a mass (14671 Da) corresponding to that of the mature avidin plus three extra amino acids from the signal peptide leader (QTV) according to design of the construct. The affinity of the avidin produced

in *E. coli* to biotin was characterised with an IAsys optical biosensor (Marttila et al. 1998) and it was comparable ($K_d \approx 4.3 \times 10^{-8}$ M) to that of commercial avidin ($K_d \approx 2.1 \times 10^{-8}$ M). The dissociation assay with fluorescent biotin (Hamblett et al. 2002) also revealed similar properties between these proteins. The stability of the protein was assayed by an SDS-PAGE-based method (Bayer et al. 1996). Both proteins showed similar high stability, which was further enhanced in the presence of biotin. Analysis of the oligomeric structure by FPLC gel filtration showed the isolated protein to have high homogenicity and to be of good quality (I, Fig. 3).

5.2 Avidin-related protein 4/5 (II)

Avidin-related protein 4/5 was selected for further study, since it exhibited promising properties in a previous study (Laitinen et al. 2002). The expression construct was mutagenised to reduce the glycosylation by mutation Asn43Glu, according to the sequence of avidin. Cysteine residue 122 was mutated to serine to block the formation of disulphide bridges between subunits from different tetramers. The proteins were produced by the baculovirus system and purified by 2-iminobiotin affinity chromatography (II, Fig. 1).

Glycosylation of the resultant proteins was analysed by treating the proteins with endoglucosidase and analysing the deglycosylated samples with SDS-PAGE (II, Fig. 3). The mutant An43Glu was found to be less glycosylated than the wt protein. This was expected owing to removal of a potential glycosylation site. Mutation Cys122Ser did not seem to have a significant effect on the glycosylation of the AVR4/5.

The biotin-binding properties of AVR4/5 were studied by measuring the dissociation constant and association and dissociation rate constants with an IAsys optical biosensor. The mutagenesis had no significant effect on the binding properties (II, Table 3). The dissociation constant for AVR4/5 forms was about one order of magnitude higher than that of avidin. Furthermore, the biotin-binding affinity of AVR4/5(C122S) was determined on the basis of the combination of ITC and DSC data as described in (Brandts & Lin 1990). The analysis revealed decrease in biotin-binding affinity of about 300-fold ($K_d \approx 3.6 \times 10^{-14}$ M) when compared to that of avidin ($K_d \approx 1.1 \times 10^{-16}$ M; II, Table 2).

Stability properties of the proteins were studied by DSC (II, Table 1) and microplate assay (II, Fig. 4). DSC analysis revealed that AVR4/5 had extremely high thermal stability (T_m = 107.4 °C). The presence of a disulphide-reducing agent, 2-mercaptoethanol, did not cause significant change in protein stability (T_m = 106.5 °C). Analogously, neither mutation Cys122Ser (T_m = 106.4 °C) nor Asn43Glu (T_m = 104.9 °C) had any significant effect on stability. The microplate assay performed on the proteins confirmed these findings, since, compared to avidin, all the AVR4/5 forms showed notably higher resistance to thermal

treatment. The AVR4/5 mutant Cys122Ser showed by far the best stability in this assay (II, Fig. 4).

5.3 Structure of AVR4/5 (III)

The structure of AVR4/5(C122S) with bound biotin was resolved by using proteins expressed both in insect cells (II) and in *E. coli* (I). Furthermore, the structure of the apoprotein was also determined from protein sample expressed in *E. coli* (III, Table 1). For some reason, structural determination of the apo form of AVR4/5(C122S) produced in insect cells was unsuccessful. The resolution obtained in the case of protein produced in insect cells was rather good (1.7 Å) and that expressed in bacteria remarkably high (1.0–1.2 Å).

The overall fold and quaternary structure of AVR4/5(C122S) are very similar to those of avidin (Livnah et al. 1993b, Pugliese et al. 1993). Four identical eight-stranded β-barrels form the tetramer (III, Fig. 5). The biotin-binding site is inside the barrel and the conformation of the biotin in the bound state is similar to that of biotin in the avidin-biotin complex (Livnah et al. 1993b). However, the valeryl acid tail of the biotin is slightly more bent in the AVR4/5(C122S)-biotin complex than in the avidin-biotin complex (III, Fig. 3B).

The residues forming direct contacts with biotin in avidin (Livnah et al. 1993b) are rather well conserved in AVR4/5 (Laitinen et al. 2002). The important hydrogen-bonding residues of avidin (Klumb et al. 1998, Marttila et al. 2003) which form contacts with biotin $O^{2'}$ are all conserved, as well as the residues forming bonds with the ureido nitrogens of biotin (Livnah et al. 1993b). The differences in the complexes can be seen in the biotin valeryl acid tail bonding residues from L3,4 (III, Fig. 4). In avidin, residues Thr38, Ala39 and Thr40 form hydrogen bonds with biotin (Livnah et al. 1993b). The L3,4 in AVR4/5 is significantly different in conformation (III, Fig 3A). The proline residue in the middle of the loop (III, Fig. 1) causes a bend in the L3,4 and the bonding to biotin shows different characteristics in this region when compared to that of avidin. Since only Asp39 forms a hydrogen bond with biotin from L3,4, the scheme resembles that found in the streptavidin-biotin complex, in where only one hydrogen bond is formed between the valeryl acid of biotin and streptavidin (Weber et al. 1989).

The interface residues of AVR4/5 are rather well conserved when compared to avidin (Livnah et al. 1993b, Laitinen et al. 2002). The difference Ile117Tyr found in the interface between subunits one and three (Livnah et al. 1993b) is hypothesised to be at least partially responsible for the better thermal stability of AVR4/5 when compared to that of avidin (II). The particular tyrosine residue in AVR4/5 forms a ring stack with another tyrosine 115 from the neighboring subunit (III, Fig 5.). Furthermore, the residue 115 forms a hydrogen bond with the lysine 92 residue from the neighboring subunit and

therefore further enhances the linkage between subunits one and three (III, Fig 5.).

Since ionic interactions have often proved to be responsible for the high thermostability of proteins (Tanner et al. 1996, Vogt et al. 1997, Vetriani 1998, Szilagyi & Zavodszky 2000, Petsko 2001), the number of salt bridges between avidin and AVR4/5 were compared. Notably, in AVR4/5(C122S), only three ionic interactions were detected per subunit. This is significantly fewer than those in avidin, which bears seven salt bridges per subunit (Livnah et al. 1993b). Streptavidin has a similar feature in so far as there are four salt bridges per subunit (Hendrickson et al. 1989, Weber et al. 1989). Therefore the main stabilizing contribution in AVR4/5 seems to be the optimisation of the structure at the interface between subunits one and three (III, Fig. 5). The different conformation of the loop between β3 and β4 seems to play a role both in stability and biotin binding (III, Fig. 3). Furthermore, the shortening of the loop between β4 and β5 might also contribute to the increased stability of AVR4/5 compared to avidin (III, Fig. 1).

5.4 Chimeric forms of AVR4/5 and avidin (IV)

In the previous study (II), AVR4/5 exhibited interesting properties, namely high biotin-binding affinity linked to extreme thermostability. This motivated us to probe further into the mechanism behind these properties. By using mutagenesis techniques and recombinant protein expression, we created different types of chimeric forms of avidin and AVR4/5 (IV, Table I).

According to the sequence alignment for avidin and AVR4/5, a segment containing residues 38-60 was observed to bear most of the significant differences between these two proteins (numbered according to avidin sequence) (IV, Fig. 1A). At first, this segment was transferred to avidin from AVR4/5. The resultant protein bears the 21-residue region from AVR4/5 in the middle of the avidin sequence, replacing the 23-residue region in the avidin sequence. This protein was named ChiAVD (IV, Fig. 1B). The second form of avidin was mutagenised by introducing potentially stabilising (II, III) point mutation Ile117Tyr into the interface between subunits one and three of avidin (AVD(I117Y)). In the third form, the above-mentioned modifications were combined in the same protein ChiAVD(I117Y). The fourth protein characterised in this study was AVR4/5(C122S) (II), which was produced in bacteria (I), thus lacking glycosylation in the three potential N-linked glycosylation sites (Laitinen et al. 2002).

The protein forms were produced by the baculovirus expression system or in *E. coli* and isolated and purified by affinity chromatography on a 2-iminobiotin column (IV, Fig. 2). Their oligomeric assembly was analysed by FPLC gel filtration analysis and proved tetrameric for all samples (IV, Table 2). The stability of the proteins was studied by DSC analysis and by a previously

described microplate assay (Nordlund et al. 2003a). In these analyses, both the sequence stretch substitution and point mutation Ile117Tyr were found to improve the stability of the protein. When these two modifications were combined in same protein, an extremely stable form of the protein was obtained, exhibiting a T_m approximately 5 °C higher than that of AVR4/5 (IV, Table II). The proteins were also subjected to proteinase K treatment, in which those bearing an AVR4/5-derived region showed resistance against proteolytic activity (IV, Fig. 3).

The biotin-binding properties of the proteins were analysed by assays with radiobiotin (IV, Fig. 4), fluorescent biotin (IV, Table 4) and an optical biosensor (IV, Table 3). These analyses showed that the different structure of the region between β3 and β5 AVR4/5 (III, IV, Fig. 6) is also reflected to the biotin-binding properties of these proteins. Avidin and AVD(I117Y) showed tighter biotin binding than the other proteins characterised (IV), supporting the previous findings regarding the lower biotin-binding affinity of AVR4/5 compared to avidin (II). The glycosylation of AVR4/5 was found to play only a minor role in the biotin binding, since non-glycosylated AVR4/5(C122S) produced in *E. coli* showed slightly tighter biotin binding when compared to the glycosylated protein produced in insect cells (IV, Fig. 4, Table 4).

A slower dissociation rate of radioactive biotin and fluorescent biotin from avidin mutant AVD(I117Y) compared to avidin was observed (IV, Table IV, Fig. 4). This somewhat surprising finding was further confirmed by competitive dissociation analysis on a 2-iminobiotin surface with an IAsys optical biosensor, using free biotin as a competitor, which showed dissociation rate constants $(12.7 \pm 0.9) \times 10^{-3}$ s^{-1} M^{-1} and $(9.9 \pm 0.8) \times 10^{-3}$ s^{-1} M^{-1} for avidin and AVD(I117Y), respectively (n = 4; Hytönen, unpublished). Together, the slower dissociation rate was observed using three different methods, and it seems to be independent of the type of biotin ligand used as a probe (free biotin (^{3}H-biotin), conjugated biotin (BF560-biotin), 2-iminobiotin surface).

Comparison of the dissociation rate constants obtained from the ^{3}H-biotin dissociation and fluorescent biotin dissociation analyses showed in the case of AVR4/5 an increase of about 6-fold in the dissociation rate of free biotin and an increase of about 3-fold in the dissociation rate of biotin-conjugate at 50 °C (IV). The ^{3}H-biotin dissociation data extrapolated to 25 °C reveals a difference of about 3-fold in the dissociation rates since the dissociation rates of the fluorescent biotins were alsmost identical in this temperature for avidin and AVR4/5 (IV, Table IV). From the biotechnological viewpoint this property of AVR4/5 and ChiAVD is interesting since the biotin is usually covalently linked to another molecule in applications.

6 DISCUSSION

6.1 Development of a bacterial expression method for chicken avidin

Chicken avidin is an efficient tool in applications in the life sciences (Wilchek & Bayer 1984, Wilchek & Bayer 1988, Bayer & Wilchek 1990, Wilchek & Bayer 1990a, Wilchek & Bayer 1990b, Wilchek & Bayer 1999). Chicken eggs provide the commonest source of avidin at present. The amount of avidin in egg-white is about 0.05% of the total protein (Green 1975). Some years ago, a powerful method of producing chicken avidin in maize was described (Kusnadi et al. 1998, Hood et al. 1999, Kramer et al. 2000). This method has been adopted on a commercial scale by Sigma (Ma et al. 2003).

Furthermore, avidin production in other eukaryotic organisms has been successful. Production in *Pichia pastoris* has yielded high amounts of secreted avidin in culture supernatant (330 mg/L) (Zocchi et al. 2003). Production in *Spodoptera frugiperda* insect cells was first shown by Airenne et al. (1997), who were able to yield milligrams of active avidin from collected insect cells per litre of culture medium. The production of chicken avidin, being a eukaryotic protein, has proven to be difficult in prokaryotes. Cytoplasmic expression of avidin in *E. coli* has produced low yields of soluble protein (Airenne et al. 1994) or avidin aggregates as inclusion bodies (Nardone et al. 1998). Therefore, an efficient method of avidin production in bacteria has been lacking.

A potential reason for the inactivity of cytoplasmic avidin is the presence of disulphide bridges. Since the redox environment of the *E. coli* cytoplasm does not support the formation of disulphide bridges, the proper folding of proteins containing disulphides is disturbed. For example, Martineau & Betton (1999) have found that an antibody fragment bearing a disulphide bridge was inefficiently produced in a cytoplasmic construct, whereas a periplasmic signal enhanced the formation of native fold. Furthermore, they found that the low yield was due to aggregation resulting from the lack of disulphide bond

formation. Their study and other studies support the hypothesis that protein aggregation is not due only to lower folding energy owing to the lack of disulphide formation (Goto & Hamaguchi 1979, Betz 1993) but also to the kinetics of the protein folding and aggregation (Kiefhaber et al. 1991, Jaenicke 1995). Notably, the production of streptavidin in an active form has been found difficult in bacteria, and therefore, production in an inactive aggregated form (inclusion bodies) followed by denaturation and renaturation is often utilised (Sano & Cantor 1990, Thompson & Weber 1993, Chilkoti et al. 1995a).

Previously, the periplasmic expression of streptavidin using a bacterial signal peptide has yielded high amounts of active streptavidin (Voss & Skerra 1997). Since streptavidin lacks a disulphide bridge, the explanation of the low yields obtained from cytoplasmic expression should lie in other properties of the protein, such as low solubility of the protein or protein intermediate. The application of a bacterial signal peptide from the OmpA protein in *Bordetella avium* bacteria (Gentry-Weeks et al. 1992) was found to be an efficient way to achieve the expression of active avidin in *E. coli* (I). The avidin thus produced was found to resemble commercial chicken avidin in its properties, showing similar biotin-binding, quaternary structure and thermostability characteristics (I, Table 1). Compared to the results obtained by the periplasmic expression of streptavidin (Voss & Skerra 1997), one may assume that these proteins share similar needs in their expression systems.

The avidin produced in the bacterial expression system lacks the carbohydrate chain. This property could offer advantages in some applications (Rosebrough & Hartley 1996). In addition, the avidin produced was efficiently crystallized under various conditions (I). This option may make it a more efficient tool for structural studies when compared to avidin produced in eukaryotic expression systems, since glycosylation has been proved to cause problems in X-ray crystallographic determination of avidin structure (Livnah et al. 1993b, Pugliese et al. 1993). Most importantly, avidin expreesion in E. coli enables avidin to be used as a basic tool in the creation of further tools for use in avidin-biotin technology, since avidin expression in bacteria is significantly easier, faster and cheaper than in eukaryotic expression systems (Airenne et al. 1997, Kramer et al. 2000, Zocchi et al. 2003). In fact, we have already produced tens of mutated avidin forms in our laboratory by using this production system.

6.2 Avidin family - structural and functional characteristics

The biological roles of avidin-related proteins are unknown. The story began when genetic material showing a relationship with avidin was detected during attempts at molecular cloning of the avidin gene (Keinänen et al. 1988, Keinänen et al. 1994). Later, two new genes were cloned by Ahlroth et al. (2000). The analysis of the genes revealed that they might be able to encode for functional proteins resembling avidin. Furthermore, they were assumed to

include functional promoter regions, since the mRNA of some of the genes had been detected during inflammation reaction (Kunnas et al. 1993).

In my undergraduate studies, the functionality of the family of AVR proteins was confirmed by protein expression in insect cells and isolating the proteins by affinity chromatography (Hytönen 2001, Laitinen et al. 2002). The proteins thus obtained were found to bind biotin with a variety of affinities ranging from avidin-like tight affinity (AVR4/5) to moderate biotin-binding affinity (AVR2). The properties of the proteins motivated us to continue working with them. We found, first, that AVR4/5 is an extremely stable protein (T_m = 107.4 °C) (II). We then studied the importance of the cysteine residue located close to the C-terminus of the protein, and found that it was not responsible for the protein's high thermal stability. Furthermore, we found that this residue causes the formation of higher-order oligomers by joining AVR4/5 tetramers together *via* disulphide bridges (II). The biotin-binding properties of AVR4/5(C122S) were found to resemble those of avidin, but the determined affinity was found to be about 300-fold lower. Altogether, the new protein showed interesting characteristics, rendering it very suitable for special (strept)avidin-biotin applications. This was also demonstrated by coating microparticles with avidin and AVR4/5 and comparing their properties at high temperature (II).

The characteristics causing the high stability of AVR4/5 were tracked using molecular modelling (II). The results suggest that only slight changes between the proteins cause the differences in their properties. Molecular modelling indicated that the most probable reasons for the higher thermal stability of AVR4/5 were Ile117Tyr difference in the one-to-three interface, a shorter L4,5 loop and the change Xaa→Pro found in L3,4 (II). Furthermore, a salt bridge was thought to form close to the carboxyl tail of biotin in AVR4/5 (II, Fig. 6). Later, analysis of the 3D structure by X-ray crystallography confirmed these ideas (III). The resolved structures of AVR4/5 show remarkably good resolution. A survey of the PDB depositions (more than 23000 structures) from international synchrotron facilities (http://asdp.bio.bnl.gov/asda/Libraries/pdb_statis/index_arch.html) in May 2005 revealed that there are fewer than 200 structures available which have a resolution of 1.0 Å or better. About 590 structures resolved at a resolution of 1.2 Å or better were found. In this light, the resolved 1.0 Å structure of AVR4/5 represents the highest resolution in protein structure research, given that less than 1% of the deposited structures attain the level of 1.0 Å.

The loop between β3 and β4 has a significantly different conformation in AVR4/5 when compared to avidin (Livnah et al. 1993b). The resulting bonding in this region with biotin is also different, resembling that of streptavidin (Weber et al. 1989) (III, Fig. 4). Comparative modelling (II) suggested that the tyrosine residue in the position corresponding to Ile117 in avidin allows bonding with adjacent tyrosine from the neighbouring subunit (III). The contact is a π-π-interaction between the benzene rings, the contact between subunits one and three being further enhanced by hydrogen bonds formed between

Tyr115 and Lys92 (III, Fig. 5C). Notably, His127 residues from the neighbouring subunits also form a π-π-interaction at the analogous interface in streptavidin (Weber et al. 1989). Furthermore, the importance of this particular histidine residue for the functionality of streptavidin has been proved by random mutagenesis experiments (Avrantinis et al. 2002).

Only a low number of ionic bonds (three) were detected in AVR4/5 (III) when compared to the seven in avidin (Livnah et al. 1993b). This is noteworthy, since ionic bonds are thought to be an important factor in the stability of proteins. For example, Bogin et al. (2002) were able to thermally stabilise the alcohol dehydrogenase by creating new salt bridges in the protein. Analogously, Vetriani et al. (1998) were able to stabilise glutamate dehydrogenase by introducing new salt bridges into the protein. Furthermore, by comparing the thermostability in 18 different protein families, Kumar et al. (2000) were able to determine that ionic interactions were more frequent in thermophilic proteins when compared to mesophilic proteins in 12 protein families. However, other studies have shown that ionic interactions do not always play a significant role in protein stability (Hendsch & Tidor 1994, Phelan et al. 2002).

In order to confirm these findings, avidin and AVR4/5 were subjected to further molecular engineering (IV). The most variable region was first substituted in avidin and the biotin-binding characteristics of the protein were found to resemble those of AVR4/5. The thermostability of the resultant protein, called ChiAVD, was found to be between avidin and AVR4/5. The importance for thermal stability of the Ile117Tyr difference at the one-to-three interface was confirmed when avidin mutant AVD(I117Y) was created. The biotin binding properties of this particular form of avidin seemed to resemble those of avidin, according to the analyses. Importantly, the dissociation rate of the radiobiotin was found to be slightly slower when compared to that of avidin and assays with fluorescent biotin as a probe supported these findings (IV).

When the above-described modifications were put together in the ChiAVD(I117Y) protein, the resultant mutant showed improved thermal stability even when compared to AVR4/5. The finding that avidin and AVR4/5 are structurally compatible enhances the hypothesis that these proteins have a close relationship, favoring the prospect of frequent recombination inside the avidin gene family looks favourable (Ahlroth et al. 2000, Ahlroth 2001, Ahlroth et al. 2001a, Ahlroth et al. 2001b). The reason that avidin is a better ”scaffold” for elements causing thermostability is not easy to determine. However, some of the sequence differences might allow good guesses. Glycosylation in Asn117 in subunit one is rather close (9.2 Å) to Asn117 in subunit three AVR4/5 (III). Therefore, one may suggest that repulsion exists between these glycosylation moieties, since the Asn117 residues point towards each other at the interface between subunits one and three. The corresponding residue in avidin is isoleucine.

A noteworthy finding in the present research effort was that we were able to decrease the dissociation rate from avidin by mutation Ile117Tyr. During the last decade of (strept)avidin modification, a similar finding has not been reported. This observation was confirmed using independent methods (IV, Hytönen unpublished). In the light of general knowledge of (strept)avidin, its tetrameric structure plays a significant role in the tight biotin binding, mainly for structural reasons since the biotin-binding site is formed of residues from two subunits (Weber et al. 1989, Livnah et al. 1993b). However, in the case of streptavidin, the dissociation of biotin is not thought to be controlled by subunit dynamics but rather by the movement of water molecules through a "water channel" simultaneously with ligand dissociation (Stayton 1999, Stayton et al. 1999b, Hyre et al. 2002). Biotin binding affinity has been found to decrease in many mutations, causing a loss in the stability of the (strept)avidin tetramer (Laitinen et al. 2001, Qureshi et al. 2001, Nordlund et al. 2003b). Our results suggest that increased oligomeric stability has a role in the biotin dissociation process (IV). The most reliable reason in decreased dissociation rate for AVD(I117Y) seems to be the combination of optimal interaction between subunits and minimal disturbance of the contact network between avidin and biotin. In this respect, the use of closely related proteins offers a powerful starting point for studies aiming at the development of protein properties. It has been previously found that thermophilic protein relatives can be used to increase the stability of mesophilic proteins (Lehmann et al. 2000, Lehmann et al. 2002).

The functional importance of AVR proteins remains an open question. The biotin-binding properties of AVRs evidently differ from those of avidin (Laitinen et al. 2002) (II). However, especially in the case of AVR4/5, the biotin-binding affinity is very high, resembling that measured for streptavidin (Green 1990). Therefore, other reasons for the use of avidin over AVRs in chicken egg-white should exist. Another evident difference between avidin and AVR proteins are the extraneous cysteine residues found in all the latter, excluding AVR2. These were found to cause intermolecular disulphide bridges (Laitinen et al. 2002) and also to link tetramers of AVRs together in higher-order oligomers (II). The biological role of these cysteines is unknown – one may assume implications for contacts with other proteins, as in the case of the oligomerization of antibodies (Sondermann & Oosthuizen 2002). When AVRs (excluding AVR2) are expressed as recombinant proteins, these residues link tetramers in solution, finally forming macroscopic aggregates (Hytönen, unpublished). These oligomeric structures can, however, easily be solubilized by adding a disulphide-reducing agent to the solution (II). Taken together, on the basis of the experimental data obtained, a schematic model of the avidin family selection can be obtained (Fig. 14). In Fig. 14, the results obtained from AVR studies are arranged in order to explain the functional properties of AVRs and the genetic events leading the current state of the family. The main hypothesis of the model is that, compared to avidin, all the AVRs found so far have problems which hinder their use. This problem could be the cysteine-

caused hyperoligomerisation in the case of AVRs, excluding AVR2. AVR2 bears the lowest biotin-binding affinity and, more interestingly, mutation Lys109Ile. This mutation, according to our preliminary results (Livnah & Hytönen, unpublished), can reduce the pseudocatalytic property of avidin (Huberman et al. 2001) and also lower the biotin-binding affinity of avidin. Again, the biological relevance of this property is not known. However, one may assume that there are reasons why AVRs are not selected for use in the chicken instead for avidin, and this mutation offers one potential explanation.

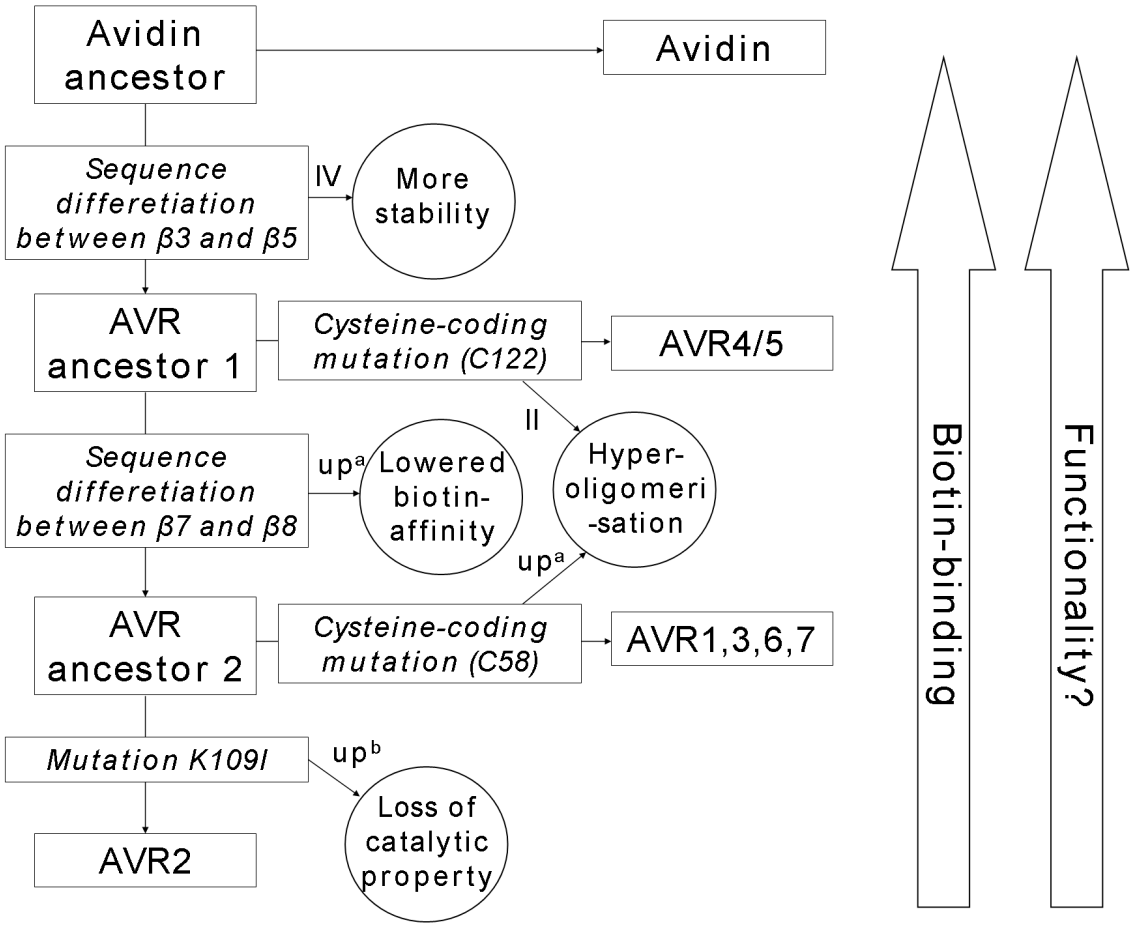

FIGURE 14 Schematic model of the hypothesis of molecular events leading to the avidin family in the chicken. The genetic differentiations are indicated by boxes leading to implications indicated by circles. Biotin-binding affinity is one measure of activity and no other measures are known. The publications in the thesis are referred by Roman numerals. up[a], Hytönen et al., unpublished results; up[b], Livnah & Hytönen, unpublished results.

Since AVR4/5 has been found to have avidin-like high biotin affinity, the reason why AVR4/5 is not used in the chicken should be something other than its biotin-binding activity. One possibility could be some problem on the genomic level leading to an unworkable promoter region, for example. However, the fact that mRNA molecules from *AVR*s have been detected under inflammation conditions in chicken tissues does not support this hypothesis (Kunnas et al. 1993). Chicken is one of the species subjected to whole-genome

sequencing (Hubbard et al. 2005). The analysis of upstream regions of *AVR* genes found in the chicken genome sequencing project showed the presence of many elements linked to functional promoters in the genes (Niskanen et al. 2005). More research is needed to explain the presence of these intact genes coding functional proteins served with functional promoter regions in the chicken genome. The explanation could be surprising, since recent findings in biotin biology research area have shown extremely interesting locations for biotin. For example, the biotinylation of histones has been proved and further characterised in several studies (Hymes et al. 1995, Stanley et al. 2001, Peters et al. 2002, Crisp et al. 2004, Narang et al. 2004).

Although AVRs are not present in egg-white in avidin-like concentrations, their expression in oviduct or other tissues cannot be excluded on the basis of current knowledge. Recent studies have suggested new roles for avidin in cell development and differentiation (Zerega et al. 2001). One may, for example, suggest that AVRs have roles in these processes, offering more variation in the biotin-binding molecules needed in developing chicken cells. A recent study threw further light on the interaction between DNA and avidin, suggesting at least partially specific interaction between these molecules *in vitro* (Morpurgo et al. 2004). If the DNA affinity for avidin has biological relevance, the scheme prompts question whether AVRs have a similar property. Since avidin and AVRs are secreted proteins, one may assume that this interaction could have a defensive role in eggs against foreign genetic material.

6.3 Biotin-binding proteins - views for research in future

Previous research on biotin-binding proteins has been largely focused on avidin and streptavidin. The biochemical properties of these proteins have been determined by several research groups using a wide variety of techniques (Chaiet & Wolf 1964, Green 1975, Gitlin et al. 1987, Hiller et al. 1987, Gitlin et al. 1988, Gitlin et al. 1989, Hendrickson et al. 1989, Weber et al. 1989, Gitlin et al. 1990, Green 1990, Livnah et al. 1993b, Pugliese et al. 1993, Gonzalez et al. 1999) and a lot of genetically modified forms of these proteins have been described (Chilkoti et al. 1995b, Chilkoti & Stayton 1995, Chilkoti et al. 1995a, Sano & Cantor 1995, Morag et al. 1996, Reznik et al. 1996, Freitag et al. 1997, Sano et al. 1997, Chu et al. 1998, Freitag et al. 1998, Klumb et al. 1998, Marttila et al. 1998, Reznik et al. 1998, Freitag et al. 1999c, Freitag et al. 1999b, Freitag et al. 1999a, Laitinen et al. 1999, Stayton et al. 1999a, Hyre et al. 2000, Marttila et al. 2000, Skerra & Schmidt 2000, Laitinen et al. 2001, Qureshi et al. 2001, Qureshi & Wong 2002, Laitinen et al. 2003, Le Trong et al. 2003, Marttila et al. 2003, Nordlund et al. 2003b, Nordlund et al. 2003a, Pazy et al. 2003, Zocchi et al. 2003, Nordlund et al. 2004, Hytönen et al. 2005, Nordlund et al. 2005b). Studies like these have sought to elucidate or modify major properties of (strept)avidin, for example ligand binding and oligomerisation. (Strept)avidin has also been

extensively modified for more specialised tasks, for example by introducing fluorescent nonnatural amino acids into the protein (Hohsaka et al. 2004). However, very few of these products have really found commercial applications – one example of new promising product in the (strept)avidin-biotin product family is StrepTactin, which is a modified streptavidin showing high affinity towards the polypeptide ligand (Korndorfer & Skerra 2002). One probable reason for this is protein expression, which has not proved possible using simple and robust methods. Many efficient protocols for production of (strept)avidin have developed during the past few years. Therefore, one may assume greater availability of commercial products in the near future (Voss & Skerra 1997, Kramer et al. 2000, Zocchi et al. 2003).

As described in the previous sections, the modification of (strept)avidin has been limited to the homotetrameric approach. This has limited the methods that can be used, since different types of (strept)avidin subunits have been obtained for the same target structure only by mixing the tetrameric proteins, thereby decreasing the resolution of the final product. More importantly, the number of distinct types of molecules in the structure of interest (for example, the surface of a particle) is not simply the result of the concentration of components, since the binding behaviour, solubility and other properties can be affected by the modification of the protein. In this sense, dual chain avidin (Nordlund et al. 2004) and single chain avidin (Nordlund et al. 2005b) offer powerful tools to further develop and minimalise the (strept)avidin technology for applications demanding high resolution on the molecular level. The design and production of dual-affinity dual chain avidins showed proof of the principle (Hytönen et al. 2005).

Biotinylation is a very important biological event, as it is used to tune central biochemical functions in the cells. Biotin is known to be linked to different types of carboxylases, decarboxylases and transcarboxylases, and other biotinylation targets have been found, like histones (Hymes et al. 1995). Biotinylation occurs in certain polypeptide signals (Samols et al. 1988, Cronan & Reed 2000). Recent studies have shown that it is possible to obtain rather short sequences which are efficiently biotinylated *in vivo* (Cronan & Reed 2000). These biotinylation sequence "tags" should offer good potential in future biochemical studies. The biotinylation process was recently made more effective by codon optimisation of the gene encoding for BirA (Mechold et al. 2005). However, the determinants of the specificity of BPL-mediated biotinylation are only partially known (Cronan & Reed 2000). One interesting result was recently obtained by Choi-Rhee et al. (2004), who found biotinylation following mutation of BirA to be less specific. The mutagenesis of BirA, leading to new sequence specificity, may therefore offer an efficient tool for the life sciences. For example, a shorter biotinylation signal should offer more feasibility for biotinylation of the target protein *in vivo*. Furthermore, since biotinylation signals are naturally present in cells, the artificial, mutated BPL/BirA combined with novel biotinylation sequence could offer a more

useful way to produce biotinylated proteins without disturbing the normal biologic machinery of the cell.

The present knowledge gained from studies concerning (strept)avidin could also be used to develop biotinylation systems. For example, just by comparing the known structures of different proteins complexed by ligands, one may find potential sites for the application of mutagenesis. The availability of biotin-detection tools and variety of modified avidins together with the development of biotinylation systems offer a potential toolpack for future studies in the life sciences.

7 CONCLUSIONS

The purpose of the present studies was to extend knowledge of the biotechnical applicability of avidin and avidin-related proteins. Along the way, a greater understanding of the molecular events leading to the special features of AVR proteins was gained. The experimentally determined structures of the AVR4/5 protein forms open a window on the importance of slight differences in protein sequence to protein function. Many interesting topics remain to be studied in relation to these proteins. The main conclusions of these studies are:

1. Avidin can be efficiently expressed in *E. coli* in active form by using a bacterial signal peptide. The protein thus obtained bears similar properties to those of avidin isolated from chicken egg-white. The method can also be used to produce AVRs and mutated forms of avidin.
2. AVR4/5 is an extremely stable protein, showing avidin-like high affinity to biotin. The cysteine residue close to C-terminus of the protein does not play an important role in the stability and biotin-binding properties of the protein. This protein may find uses in applications requiring high stability.
3. The atomic resolution structure of AVR4/5 provides information about the molecular interactions leading to the special properties of AVR4/5. Only slight structural changes account for the significantly different properties of AVR4/5 and avidin.
4. Structural elements responsible for the high stability of AVR4/5 can be substituted in the avidin. Analogously, a highly divergent region between β3 and β5 in AVR4/5 also includes the structural elements responsible for the biotin binding characteristics of AVR4/5.
5. AVRs seem to fulfil the characteristics of active proteins. More research is needed to reveal the biological significance of *AVR*s.
6. AVRs can be used as a source in combinatorial protein chemistry in order to develop more powerful tools for biotin technology.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Avidiiniproteiiniperhe – perheen jäsenten ominaisuuksia ja uusia biotiinia sitovia proteiinityökaluja

Tässä tutkimuksessa keskityttiin kanan avidiini-proteiinin ja samaan geeniperheeseen kuuluvien avidiinien kaltaisten geenien (engl. avidin-related genes, *AVR*) koodaamien AVR-proteiinien ominaisuuksien tarkasteluun. Avidiinia esiintyy luonnossa kananmunan valkuaisessa, jossa sen arvellaan toimivan puolustusproteiinina suojaamassa kehittyvää alkiota esimerkiksi mikrobien toiminnalta. Tätä proteiinia on tutkittu intensiivisesti ja siitä on tuotettu rekombinanttisesti useita erilaisia muotoja. Avidiinia ja sen bakteerianalogiproteiinia, streptavidiinia, käyetään lukuisissa biotieteiden sovelluksissa.

Tässä tutkimuksessa kehitettiin uusi tapa tuottaa tehokkaasti avidiinia bakteerisoluissa. Kehitetty tuottosysteemi mahdollistaa avidiinien halvan tuotannon, ja systeemin käyttö on helpompaa moniin muihin käytössä oleviin menetelmiin verrattuna. Systeemin perusidea on se, että avidiinien alkuperäinen solun ulkopuolelle erityksessä tarvittava signaalipeptidi on korvattu bakteerin proteiinista peräisin olevalla erityssignaalilla. Näin bakteerisolun tuottama rekombinanttinen avidiini erittyy alkuperäistä huomattavasti tehokkaammin bakteerisolun periplasmiseen tilaan, joka myös mahdollistaa proteiinissa olevien disulfidisidosten muodostumisen. Tehokas tuottosysteemi myös helpottaa tämän proteiiniperheen parissa tehtävää tutkimustyötä.

Tutkimuksen muut osatyöt käsittelevät avidiinin kaltaisia proteiineja. Näistä ehkä mielenkiintoisimmaksi on osoittautunut AVR4/5, joka on erittäin termostabiili proteiini. Olemme onnistuneet selvittämään proteiinin lämmönkestävyyden aikaansaavia rakenteellisia alueita ja yksityiskohtia kohdennettuun mutageneesiin perustuvien menetelmien avulla. Tuottamalla erilaisia muokattuja muotoja kyseisestä proteiinista, olemme havainneet, että stabiilisuus ei merkittävästi selity proteiinin C-terminaalisen kysteiinitähteen olemassaololla. Samoin proteiinin glykosylaatiolla ei ole havaittu olevan keskeistä merkitystä sen suurelle kestävyydelle. Proteiinin kolmiulotteisen rakenteen määrittäminen paljasti, että kyseinen proteiini muistuttaa voimakkaasti sukulaistaan avidiinia. Keskeisimmäksi proteiinin lämmönkestävyyden lisääjäksi arvioitiin määritetyn rakenteen perusteella alayksikköjen välisen rajapinnan rakenne, joka mahdollistaa AVR4/5 proteiinissa alayksikköjen tiukemman sitoutumisen toisiinsa avidiiniin verrattuna. Myös neljännessä osatyössä proteiinin mutageneesillä hankittu kokeellinen tieto tukee tätä teoriaa. Lisäksi havaitsimme, että avidiini ja AVR4/5 ovat keskenään "yhteensopivia", sillä kykenimme rakentamaan näiden proteiinien kimeerisen muodon, jossa osa avidiinia on korvattu AVR4/5-proteiinista peräisin olevalla polypeptidillä.

Kokonaisuudessaan työ tarjoaa lisää tietoa proteiiniperheen jäsenten rakenteen ja toiminnan välisestä suhteesta ja siten avaa uusia mahdollisuuksia ja

aineistoa proteiinien muokkaamiseen ja vertailuun. Osa tutkimuksen havainnoista saattaa päätyä avidiini-biotiini teknologian työkaluiksi.

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