JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 126

Henri Nordlund

Avidin Engineering; Modification of Function, Oligomerization, Stability and Structure Topology

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Ylistönrinteen salissa (FYS3)
marraskuun 28. päivänä 2003 kello 12.

Academic dissertation to be publicly discussed, by permission of
the the Faculty of Mathematics and Science of the University of Jyväskylä,
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UNIVERSITY OF JYVÄSKYLÄ

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Publishing Unit, University Library of Jyväskylä

URN:ISBN 9513916502
ISBN 951-39-1650-2 (PDF)

ISBN 951-39-1505-0 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä
and ER-Paino Ky, Lievestuore 2003

ABSTRACT

Nordlund, Henri
Avidin engineering; modification of function, oligomerization, stability and structure topology
Jyväskylä, University of Jyväskylä, 2003, 64 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701; 126)
ISBN 951-39-1650-2
Yhteenveto: Avidiinin toiminnan, oligomerisaation, kestävyyden ja rakenne-topologian muokkaaminen
Diss.

Avidin is a basic and rather stable tetrameric glycoprotein found in chicken egg-white. It has an enormous affinity toward biotin (vitamin H), which forms the basis of so called avidin-biotin technology. This extraordinary protein-ligand pair is superior to many other protein-ligand systems because it is very robust and can be used under even harsh experimental conditions. In addition, several biotin derivatives and conjugates are commercially available.

Our main objective was to create new avidin tools with novel properties for avidin-biotin technology which would have improved performance in some applications and even open completely new possibilities in others. In addition we obtained valuable basic information about avidin. We modified avidin by the means of protein engineering using a rational mutation design strategy.

We succeeded in engineering an avidin with enhanced thermal stability characteristics by introducing cysteine residues to the monomer-monomer interfaces, which formed covalent intermonomeric disulfide bridges. We showed also that the tetrameric quaternary structure of avidin can be broken by only two crucial point mutations in the interface residues and that the resultant monomeric avidin was biologically active in the sense of biotin binding. In addition, we prepared two different circularly permuted avidins and their fusion. The resultant pseudo-tetrameric fusion avidin, in which half of the binding sites can be further modified independently of the other half, has enormous potential in new applications. Finally, we introduced histidine residues into the monomer-monomer interfaces, which led to some of the mutants becoming pH dependent with respect to biotin binding and oligomerization.

Key words: Avidin-biotin technology; protein engineering; thermal stability.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which in the text will be referred to by their Roman numerals:

I Nordlund, H. R., Laitinen, O. H., Uotila, S. T., Nyholm, T., Hytönen, V. P., Slotte, J. P. & Kulomaa, M. S. 2003. Enhancing the thermal stability of avidin. Introduction of disulfide bridges between subunit interfaces. J. Biol. Chem. 278: 2479-2483.

II Laitinen, O. H., Nordlund, H. R., Hytönen, V. P., Uotila, S. T., Marttila, A. T., Savolainen, J., Airenne, K. J., Livnah, O., Bayer, E. A., Wilchek, M. & Kulomaa, M. S. 2003. Rational design of an active avidin monomer. J. Biol. Chem. 278: 4010-4014.

III Nordlund, H. R., Laitinen, O. H., Hytönen, V. P., Uotila, S. T., Porkka, E. & Kulomaa, M. S. 2003. Construction of a dual-chain pseudo-tetrameric chicken avidin by combining two circularly permuted avidins. Manuscript.

IV Nordlund, H. R., Hytönen, V. P., Laitinen, O. H., Uotila, S. T., Niskanen, E. A., Savolainen, J., Porkka, E. & Kulomaa, M. S. 2003. Introduction of histidine residues into avidin subunit interfaces allows pH dependent regulation of quaternary structure and biotin binding. FEBS Lett. In press.

RESPONSIBILITIES OF HENRI NORDLUND IN THE ARTICLES OF THIS THESIS

Article I: I am mainly responsible for this study. However, Thomas Nyholm performed the DCS measurements, Vesa Hytönen conducted the IAsys measurements and Sanna Uotila did part of the practical work under my supervision.

Article II: Olli Laitinen is mainly responsible for this study. I am responsible for the HPLC analysis. In addition, I participated in construction of the recombinant baculoviruses, protein purification, planing of the characterisation experiments and writing the article.

Article III: I started this project together with Olli Laitinen by designing the constructs at protein level. I am mainly responsible for the subsequent steps in this study. Sanna Uotila and Eevaleena Porkka performed part of the practical work under my supervision and Vesa Hytönen contributed significantly to the protein characterisations.

Article IV: I am mainly responsible for this study. However, Vesa Hytönen is responsible for the fluorescence and IAsys studies and Janne Savolainen participated in the fluorescence studies. Sanna Uotila and Eevaleena Porkka did part of the practical work under my supervision.

All of these studies were carried out under the supervision of Professor Markku Kulomaa.

Abbreviations

3D	three-dimensional
ADP	adenosine diphosphate
AVR	chicken avidin-related gene
AVR	*AVR* encoded protein
BBP	biotin binding protein
BNP	biotinyl p-nitrophenyl ester
BPL	biotin protein ligase
CD4+	cluster determinant on T lymphocyte surface
CDR	complementarity determining region
Cpm	counts per molecule
C-terminus	carboxy terminus of a peptide
DSC	differential scanning calorimetry
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMBOSS	european molecular biology open software suite
FABP	fatty acid binding protein
FCS	fluorescence correlation spectroscopy
FPLC	fast protein liquid chromatography
F_V	variable fragment of an antibody
GCG	genetics computer group
HABA	2-(4´-hydroxyazobenzene) benzoic acid
HPLC	high pressure liquid chromatography
I-FABP	rat intestinal fatty acid binding protein
K_d	dissociation constant
kD	kilo dalton
M	molar concentration
N-terminus	amino terminus of a peptide
PCR	polymerase chain reaction
pI	isoelectric point
RBP	retinol binding protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
T_m	transition temperature midpoint of heat denaturation
V_H	variable part of an immunoglobulin heavy chain
V_L	variable part of an immunoglobulin light chain

1 INTRODUCTION

Chicken egg-white avidin is a homotetrameric glycoprotein that displays extremely high affinity toward the vitamin biotin (Green 1975). This non-covalent protein ligand interaction is one of the strongest known in nature and forms the basis of so called avidin-biotin technology, which includes several life science applications, such as targeting, labeling and purification of various substances (Wilchek & Bayer 1990, Wilchek & Bayer 1999). In addition to the value of avidin in applications, it provides an excellent model for understanding the structure-function relationship behind the high affinity ligand binding. Moreover, because avidin is composed of four identical subunits it also serves as a resource for better understanding of protein-protein interactions in general. Genetic engineering of avidin can therefore both produce valuable basic information and provide more versatile tools for avidin-biotin technology.

Premises in avidin engineering and characterisation of the mutants are the availability of the cDNA (Gope et al. 1987), the fact that the three-dimensional structure is known (Livnah et al. 1993a, Pugliese et al. 1993, Pugliese et al. 1994), the existence of an enormous amount of avidin characterisation data (Wilchek & Bayer 1990 and references therein) and the establishment of a heterologous production system in insect cells (Airenne et al. 1997). In this study we aimed to investigate the importance of the intrachain disulfide bridge of avidin (I) by replacing the wild-type cysteines with the same amino acids that streptavidin, a bacterial analogue of avidin, bears in equivalent positions (Hendrickson et al. 1989, Weber et al. 1989). In addition, the sequence information obtained from sea urchin fibropellins (Bisgrove et al. 1991, Bisgrove & Raff 1993) was utilised to design intermonomeric disulfide bridges into avidin together with the results obtained previously for a streptavidin mutant with intermonomeric disulfide bridges (Reznik et al. 1996) in order to find out if functional and even more stable avidin can be produced with this strategy (I). Moreover, on the basis of previous results with modified avidins (Laitinen et al. 1999, Laitinen et al. 2001), we were interested in studying whether a combination of two central interface mutations would lead to monomeric biotin binding avidin (II). More radical

modifications, including fusion of the original N- and C-termini and introduction of the new termini into selected positions in the β-barrel structure, were used in the creation of two circularly permuted avidins and their fusion, a dual-chain avidin (III). Finally, we introduced histidine residues into the monomer-monomer interfaces and succeeded in converting avidin into a pH-sensitive form in respect of ligand binding and oligomerization (IV).

2 REVIEW OF THE LITERATURE

2.1 Avidin

The name avidin was given in early 1940s to a minor chicken egg-white protein, which displayed a peculiar biotin (vitamin H) binding capacity (Eakin et al. 1941). Before that, the nutritional effects of ingested raw egg-white had been studied (Steinitz 1898, Bateman 1916, Boas 1927) and the reason for the toxicity was found to be a protein constituent that made biotin unavailable to organisms. The first attempts to purify avidin, based on selective solubilization by salt from alcohol-precipitates (Eakin et al. 1941) produced only 30 % active avidin preparations. More effective procedures were developed and avidin samples suitable for thorough biochemical characterization were obtained and accumulation of the data concerning avidin as a protein really began (Green 1975 and references therein). Besides chicken egg-white, in which avidin represents 0.05 % of total protein, avidin has been found to be present in the egg-white of several other birds, reptiles and amphibia (Hertz & Sebrell 1942, Botte & Granata 1977, Korpela et al. 1981, Robel 1987). Chicken oviduct produces avidin upon induction by the steroid hormone progesterone (O'Malley & McGuire 1969). In addition to that, avidin is produced in several other tissues as an inflammatory response protein (Tuohimaa et al. 1989 and references therein). One important landmark in the history of avidin was the elucidation of its amino acid sequence (DeLange & Huang 1971) as well as molecular cloning of the cDNA (Gope et al. 1987) and the gene (Wallén et al. 1995).

2.1.1 General properties

Avidin is a basic (pI ≈ 10.5) homotetrameric glycoprotein composed of four identical subunits. Each subunit includes 128 amino acids and the tetramer binds biotin with the strongest non-covalent affinity interaction known in nature between a protein and a ligand ($K_d \approx 10^{-15}$ M). The molecular weight of

the tetramer is around 63 kDa and the sugar moiety attached to asparagine 17 in each subunit comprises 10 % of the weight and exhibits some microheterogeneity (Green 1975, Bruch & White 1982). Avidin is an extremely stable protein and withstands extreme pH, elevated temperatures, presence of chemical denaturants and activity of proteolytic enzymes (Green 1975, Green 1990). The avidin gene contains four exons and three introns (Wallén et al. 1995). The coding region begins with a leucine-rich signal sequence 24 amino acid residues long, which directs the nascent avidin peptide into the secretion route and is cleaved off by signal peptidase. Glycosylation and formation of the intramolecular disulfide bridge between the cysteine residues 4 and 83 takes place in the secretion route (Green 1975, Gope et al. 1987).

2.1.2 Structure and function

The three-dimensional crystal structure of avidin as a complex with biotin (Livnah et al. 1993a, Pugliese et al. 1993) and without the ligand (Livnah et al. 1993a, Pugliese et al. 1994) was obtained after many attempts to grow suitable crystals for X-ray crystallography (Green & Joynson 1970, Pinn et al. 1982, Gatti et al. 1984). Most of the crystals suffered from heterogeneity of the sugar moiety. However, after a partial deglycosylation procedure the crystallization was successful. Each avidin monomer is composed of eight successive β-strands and their interconnecting loop regions. The strands form an anti-parallel (up-and-down) β-barrel, in which strand 1 forms hydrogen bonds with strand 8 (Fig. 1). The N and C termini are located near each other in the non biotin-binding end of the barrel. Couple of the N- and C-terminal residues are absent from the crystal structures because of their disordered nature and consequent poor diffraction properties (Livnah et al. 1993a).

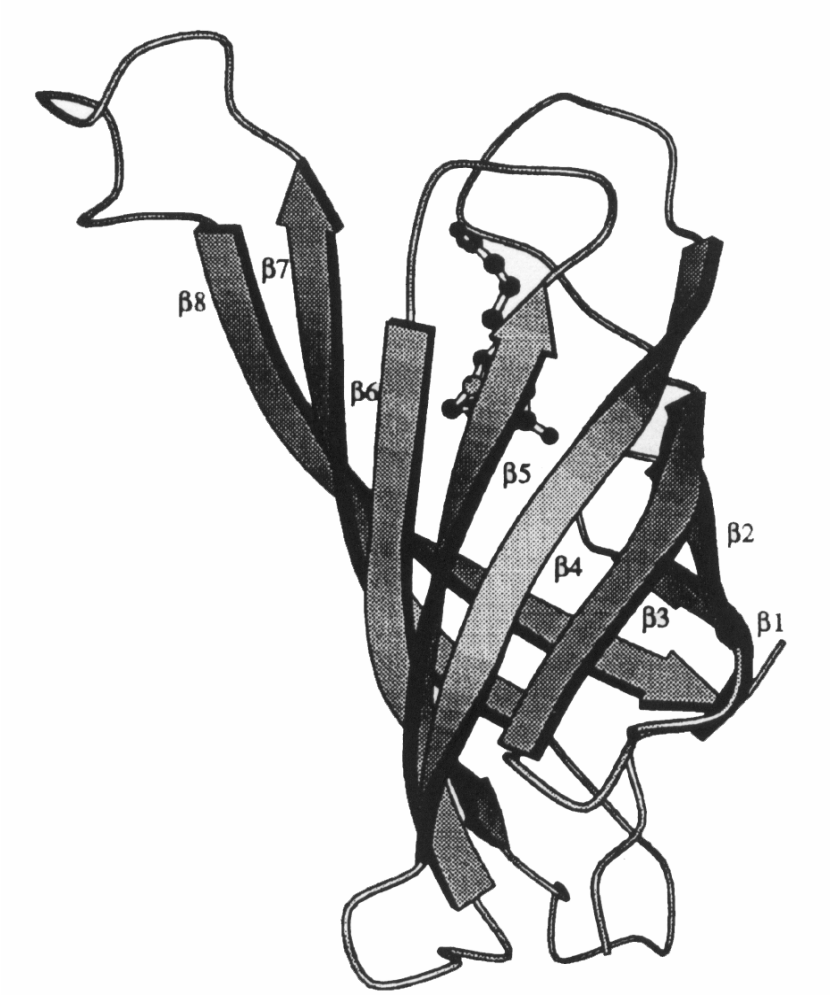

FIGURE 1 A schematic arrow representation of the avidin monomer with biotin shown as a ball and stick model in the upper part of the picture. The β-strands of the anti-parallel β-barrel are labeled from β1 to β8. The figure was reconstructed from Livnah et al. (1993a).

The amino acid residues responsible for biotin binding were precisely identified from the 3D-structure of the avidin biotin complex (Fig. 2) and the molecular basis of the extremely tight binding was understood at the atomic level as a network of hydrogen bonds and hydrophobic interactions. Comparison of the avidin structure with and without biotin also revealed that a small conformational change in the loop between β-strands three and four follows biotin binding. In addition, the peculiar biotin-binding residue tryptophan 110 that participates in the biotin binding of the neighbouring subunit becomes more inflexible and forms a lid to the biotin-binding pocket. Biotin fits into this pocket nearly perfectly and this structural complementarity is believed to be the most important factor behind their extreme affinity. The binding pocket of apo-avidin, i.e. avidin without biotin, captures five water molecules. The ordered pattern of these waters resembles the structure of biotin and hence they were originally considered to be contaminant biotin molecules in the protein crystals (Wilchek & Bayer 1999).

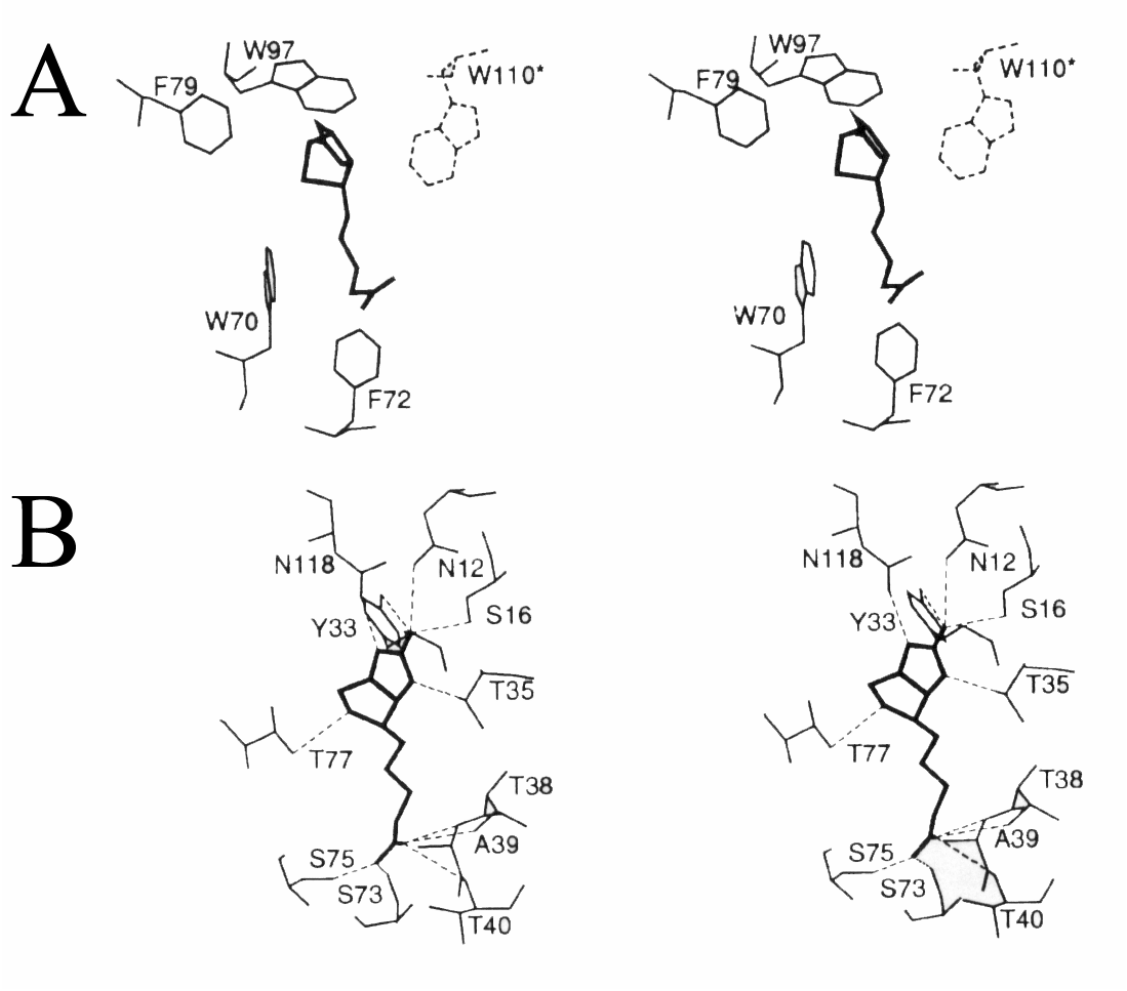

FIGURE 2 Biotin-binding site residues of avidin. (A) Hydrophobic residues including W70, F72, F79, W97 and W110. The latter, represented by dashed lines, is provided by the adjacent monomer. (B) Hydrogen bond interactions at the avidin binding site. The figure was reconstructed from Livnah et al. (1993a).

The exact interactions between the four subunits that form the tetramer (Fig. 3) were defined according to the crystal structure at the atomic level as three different types of monomer-monomer contact patterns. The one-to-two interface (and the equivalent interface three-to-four) is a function-related interface, because of the aforementioned biotin-binding interplay between the monomers *via* trypthophan 110 (Fig. 4). Therefore the one and two subunit pair is sometimes called as the functional dimer. The one-to-three interface (Fig. 5) (identically two-to-four) and the one-to-four (Fig. 6) (identically two-to-three) interface contribute to the stability of the tetrameric quaternary structure. The one and four subunit pair (and two and three) is called as the structural dimer due to its extensive monomer-monomer interactions. In addition to the tetrameric quaternary structure avidin forms higher molecular weight oligotetrameric structures, but the cause of this behavior remains unresolved (Bayer et al. 1996).

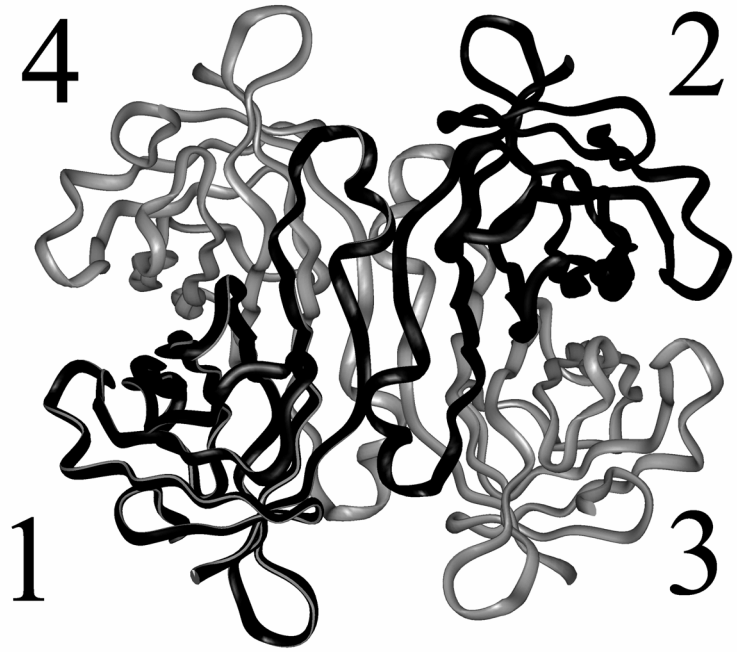

FIGURE 3 A ribbon representation of the avidin tetramer. The subunits are numbered according to Livnah et al. (1993a).

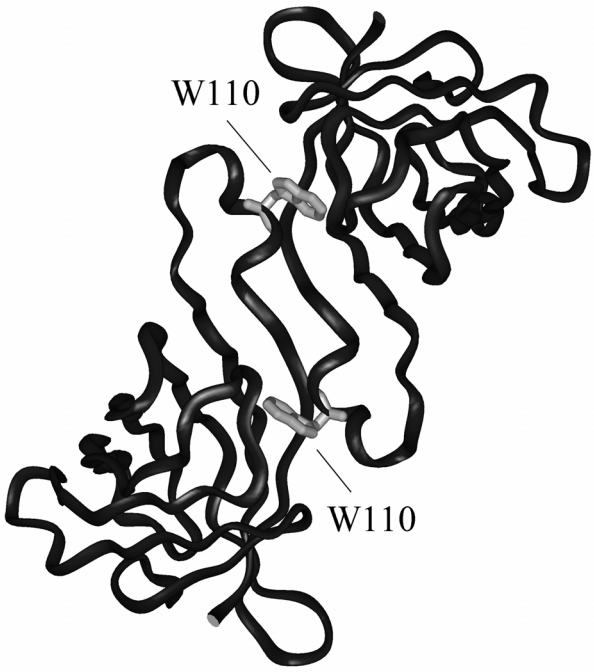

FIGURE 4 Amino acid residues involved in the one-to-two (and three-to-four) monomer-monomer interface are W110, T113 and V115. These monomers share a 729 Å^2 buried surface area as measured from the avidin-biotin complex (Livnah et al. 1993a). The side chain of W110, which participates in the biotin binding of the neighbouring subunit, is shown in the picture.

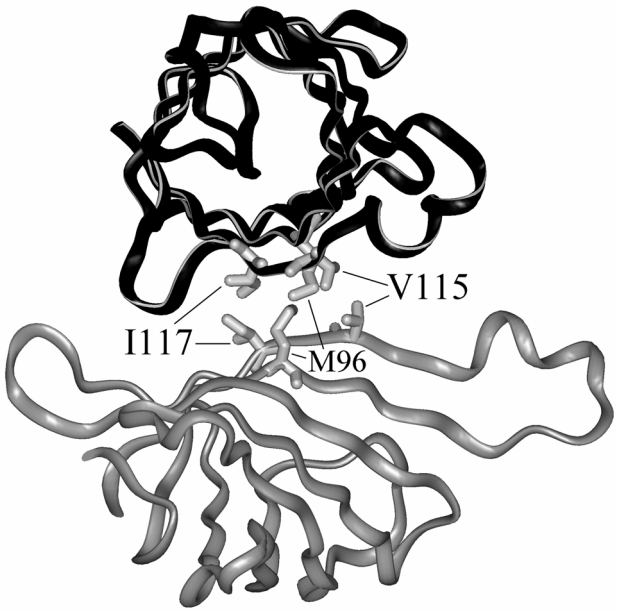

FIGURE 5 The one-to-three (and two-to-four) monomer-monomer interaction is mediated by the side chains of M96, V115 and I117 shown in the picture. Each of these amino acids faces the same amino acid from the neighbouring subunit. The buried surface area between these monomers is 120 Å^2 (Livnah et al. 1993a).

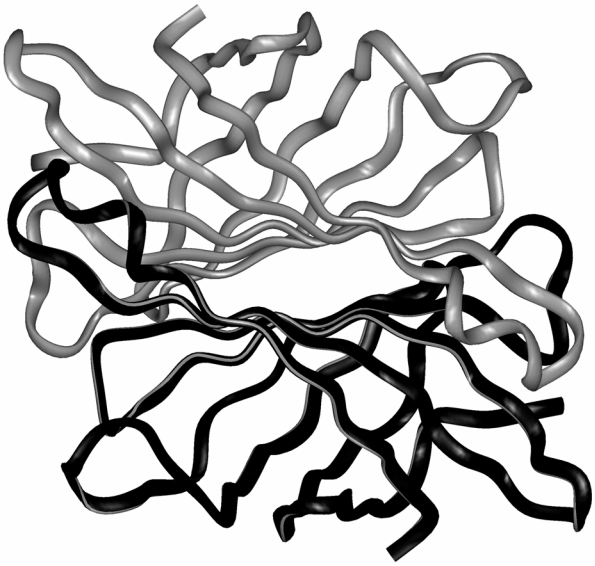

FIGURE 6 The one-to-four (and two-to-three) monomer-monomer interaction is extensive and involves the following amino acids: H50, E53, N54, T55, N57, R59, G65, T67, N69, W70, K71, S73, V78, T80, Q82, E91, M96, L98, R100, S101, V103 and T113. The buried interface area is 1951 Å^2 (Livnah et al. 1993a).

Chicken egg-white contains several anti-bacterial proteins such as lysozyme, flavin-binding protein and conalbumin. Avidin also acts as an antibacterial agent by depriving free biotin from the environment and thereby making it unavailable to bacteria. Therefore it is straightforward to think of avidin as having an antibacterial biological function, although the evidence is circumstantial only (Green 1975). There has also been evidence that avidin has an influence on cell differentiation (Zerega et al. 2001). Avidin is physiologically expressed in chicken chondrocytes and myoblasts at their late differentiation stages, and therefore it could affect cell membrane building and signal transduction indirectly through biotin-dependent fatty acid biosynthesis. In addition, it has been shown that avidin displays pseudo-catalytic activity in biotinyl p-nitrophenyl ester (BNP) alkaline hydrolysis. Studies with an amide analogue of the ester (biotinyl p-nitroanilide) suggested that the features of avidin responsible for the hydrolytic activity are the flexible nature of the loop between β-strands three and four, presence of arginine 114 and interaction of the substrate with lysine 111 (Huberman et al. 2001). Since the biotin part of the biotinyl ester is virtually irreversibly bound to avidin, the catalytic reaction proceeds in one direction without any turnover, unlike normal enzymatic reactions.

Avidin binds to a variety of different biotin derivatives and analogues (Green 1975 and references therein, Dixon et al. 2002). In addition to that it can bind different dye-molecules and peptides, which structurally may be quite distant from biotin (Gissel et al. 1995, Ostergaard et al. 1995). One of the dyes, 2-(4´-hydroxyazobenzene) benzoic acid (HABA), can be used in a colorimetric assay to measure avidin amount and activity (Green 1970). Interestingly, the colour of HABA is red-shifted and changes from yellow to red when it is bound to avidin. Upon binding HABA replaces the five water molecules from the binding site in a manner similar to that of biotin. The binding is however much weaker ($K_d \approx 6 \times 10^{-6}$ M) and therefore biotin readily replaces HABA at the binding site (Green 1990, Livnah et al. 1993b).

2.1.3 Relatives of avidin

Several proteins resemble avidin functionally and/or structurally in the sense that they bind biotin or bear some level of sequence or structural similarity. Besides proteins, *in vitro* generated RNA aptamer molecules are also known to interact with biotin.

2.1.3.1 Avidin-Related Proteins (AVRs)

The avidin gene belongs to a gene family located in the chicken sex-chromosome Z. In addition to the avidin gene this locus contains avidin-related genes (*AVRs*), the closest relatives of avidin. The number of *AVR* genes varies between individuals and seven different *AVR* genes have been described (Keinänen et al. 1988, Keinänen et al. 1994, Ahlroth et al. 2000). *AVR* genes contain putative promoter sequences and polyadenylation signals, indicating

that they could be functional. In addition to that, mRNAs for AVRs 2 and 3 have been detected (Kunnas et al. 1993), but there is no evidence of production of the AVR proteins *in vivo* in chicken. Amino acid sequences of the putative AVR proteins bear 85-100% identity with each other and 74-81% with avidin. In addition to the high degree of similarity, the amino acids involved in plausible biotin binding are even more conserved, suggesting that they might be capable of biotin binding. This was confirmed in a recent study, where recombinant AVRs were produced in insect cells and their properties were characterised and compared to those of avidin (Laitinen et al. 2002).

2.1.3.2 Bacterial streptavidin

Streptavidin, a bacterial analogue of chicken avidin was found accidentally when new antibiotics were screened (Chaiet et al. 1963, Stapley et al. 1963). Streptavidin from *Streptomyces avidinii* is produced as a preprotein 159 amino acids long, which is processed proteolytically from both termini during and after its natural secretion and the final product is 125-127 amino acids long so-called core streptavidin (Bayer et al. 1987, Pähler et al. 1987).

Streptavidin has only a moderate sequence identity (30%) and similarity (41%) to avidin (Livnah et al. 1993a). However, comparison of the 3D structure of streptavidin (Hendrickson et al. 1989, Weber et al. 1989) with that of avidin revealed that the secondary, tertiary and quaternary structures are well conserved and resemble each other in number of ways. The overall fold of a streptavidin monomer is an antiparallel eight-stranded β-barrel similar to that of avidin, the major differences residing in the loop regions, such as the shorter 3-4 loop in streptavidin compared to that of avidin. The monomer-monomer interactions in the tetrameric quaternary structure are also notably similar in interfaces one-to-two and one-to-four, whereas the one-to-three interface is somewhat different (Livnah et al. 1993a).

Streptavidin has slightly lower affinity for biotin than avidin, which is probably explained by the minor differences observed in the biotin binding sites (Green 1990). On the contrary, streptavidin appears to display higher affinity toward biotin molecules which have additional groups at the valeryl carboxylate moiety than avidin. This may indicate that streptavidin is more useful in applications where tight binding of biotinylated substances is essential (Pazy et al. 2002). In addition to that, streptavidin differs from avidin, since its pI is slightly acidic and it is not glycosylated like basic avidin (Green 1975). Streptavidin is also devoid of cysteines (Argarana et al. 1986), and therefore the intramonomeric disulfide bridge characteristic of avidin (DeLange & Huang 1971, Gope et al. 1987) is not present in streptavidin. From the thermal stability point of view streptavidin is less stable than avidin. For streptavidin the transition temperature midpoint (T_m) of heat denaturation is 75.5 °C in the absence and 112.2 °C in the presence of biotin, whereas for avidin these values are 83.8 °C and 117 °C, respectively (Gonzales et al. 1999). Furthermore, streptavidin lacks the pseudo-catalytic activity of avidin mentioned previously and, quite on the contrary, protects the biotinyl p-nitrophenyl ester from

alkaline hydrolysis (Huberman et al. 2001). Furthermore, both of the proteins bind the aforementioned color HABA, but avidin binds it with an affinity about 20-fold higher (Green 1990). Avidin and streptavidin also show different profiles of tissue uptake, organ distribution and blood clearance properties upon administration *in vivo* (Schechter et al. 1990, Schechter et al. 1996).

The closest known relatives of streptavidin from *S. avidinii* are two other streptavidins named as streptavidin v1 and v2, which were found in two strains of *S. venezuelae* (Bayer et al. 1995). The deduced amino acid sequence of streptavidin v1 is almost identical with that of streptavidin from S. avidinii, displaying only alanine 100 to threonine substitution. On the other hand, streptavidin v2 contains nine substitutions. Only five of these are located in the core region and they do not involve biotin-binding residues. It is possible that there are several other bacterial streptavidins, since in the original work (Stapley et al. 1963) eleven additional bacterial strains were described to have antibiotic activity similar to that of *S. avidinii*.

More recently a putative (strept)avidin-like protein has been found in a comprehensive whole genome sequencing project concerning a nitrogen-fixing symbiotic bacterium, *Bradyrhizobium japonicum* (Kaneko et al. 2002). This bacterium is very important agriculturally, since it can form root nodules on soybeans. The overall sequence identity of the *B. japonicum* avidin with chicken avidin and streptavidin is around 30 % and similarity around 40 %. More importantly, from the putative biotin-binding residues nine to ten (depending on the alignment parameters) out of 12 are conserved when compared to those of streptavidin. Furthermore, when compared to chicken avidin the result is seven out of 16, which is interestingly very close to the biotin-binding residue identity between avidin and streptavidin.

2.1.3.3 Other biotin binders

In addition to avidin, chicken egg contains two other biotin-binding proteins called BBP-I and BBP-II (White & Whitehead 1987). The molecular weight of BBP-I in denaturing conditions is around 68 kD, whereas BBP-II has a molecular weight roughly one quarter of that figure (18 kD). Antibodies elicited against BBP-II cross-react readily with BBP-I. The N-terminal sequences of these two proteins are markedly similar and interestingly also display slight similarity with avidin, suggesting some level of evolutionary relationship (Bush & White 1989). Combined, these results also indicate that BBP-II is most probably a proteolytically cleaved form of BBP-I. Therefore, BBP-I is a monomeric protein with four tandem repeats of biotin-binding domains, and BBP-II is a tetrameric biotin-binder with four identical or nearly identical subunits. The majority of the biotin in egg-yolk is bound to biotin-binding proteins. Both BBP-I and BBP-II circulate in the bloodstream and bind biotin from plasma. After that, these BBP-biotin complexes are transported to the ovary and finally deposited into the developing oocyte, where they are act as biotin suppliers for the developing embryo (White & Whitehead 1987).

Monoclonal antibodies with relatively high affinity toward biotin ($K_d \approx 10^{-9}$ M) have been described (Bagci et al. 1993). In that study mice were immunized with biotinylated BSA and the clones were screened according to their biotin-binding properites. The cDNA of the V_H (Bagci et al. 1993) and V_L (Kohen et al. 1997) parts of the anti-biotin antibody were cloned and sequenced. Interestingly, the CDR2 and CDR3 regions of the V_H part showed some resemblance to the biotin-binding sites of avidin and streptavidin, in particularly with β-strands three and six. On the other hand, the V_L part showed no significant resemblance with avidin or streptavidin.

Ligand-binding RNAs and DNAs are called aptamers. A biotin-binding aptamer with a moderate affinity to biotin (around 6×10^{-6} M) was generated in an *in vitro* evolution study, in which biotin-binding RNA molecules were screened from a pool of random RNA sequences (Wilson & Szostak 1995). The fact that more than 90% of the obtained biotin-binding aptamers were identical indicated that there are only few molecular solutions capable of producing a strong RNA-biotin interaction. The 3D structure of the aptamer (Nix et al. 2000) confirmed the predicted structural resemblance (Wilson et al. 1998) to retroviral frame-shifting elements (Chen et al. 1995), which fold as RNA structures called pseudoknots. The aptamer-biotin interaction is markedly different from that of avidin-biotin. It buries only part of the biotin in a relatively solvent-accessible pocket and the valeryl moiety of biotin remains largely exposed. This is explained by the fact that the aptamers were screened originally with biotin molecules immobilised via the valeryl carboxylate, and sterical hindrance may have prevented the interactions with this part of biotin. The polar interactions with the ureido ring of biotin are formed in avidin by amino acid side-chain hydroxyl and amide-groups, whereas in the biotin aptamer these interactions are mediated by solvent cofactor magnesium ions and structured water molecules. Although the interaction of the aptamer-biotin complex is about nine orders of magnitude weaker than that of avidin-biotin, it is noteworthy that it is within an order of magnitude of that of the immobilised monomeric avidin-biotin interaction (Kohanski & Lane 1990).

Avidin and all of the biotin-binding proteins and molecules described in the previous sections bind biotin with variable affinities and mechanisms, but share the fact that the binding is non-covalent. Biotin-dependent carboxylases and transcarboxylases, however, contain covalently bound biotin, as a prosthetic group (Campeau & Gravel 2001 and references therein). These enzymes catalyse the key steps of gluconeogenesis, lipogenesis, amino acid degradation, and energy transduction (Samols 1988). Biotin is attached to these enzymes during posttranslational modification catalysed by biotin protein ligase (BPL), which couples biotin covalently to a lysine ε-amino group in a conserved target sequence.

2.1.3.4 Similar super secondary structures

The avidin and streptavidin fold is an antiparallel β-barrel composed of eight successive strands with a repeated +1 topology representing thereby the simplest possible topology for a closed, barrel-like β-sheet (Fig. 7). This kind of super secondary structure is not exclusive to (strept)avidins and can also be seen in a protein family called lipocalins and, taking a slightly broader view, in fatty-acid-binding proteins (FABPs). Together these three ligand-binding protein families of avidins, lipocalins and FABPs form a part of an overall structural superfamily, the calycins (Flower 1993, Flower et al. 1993).

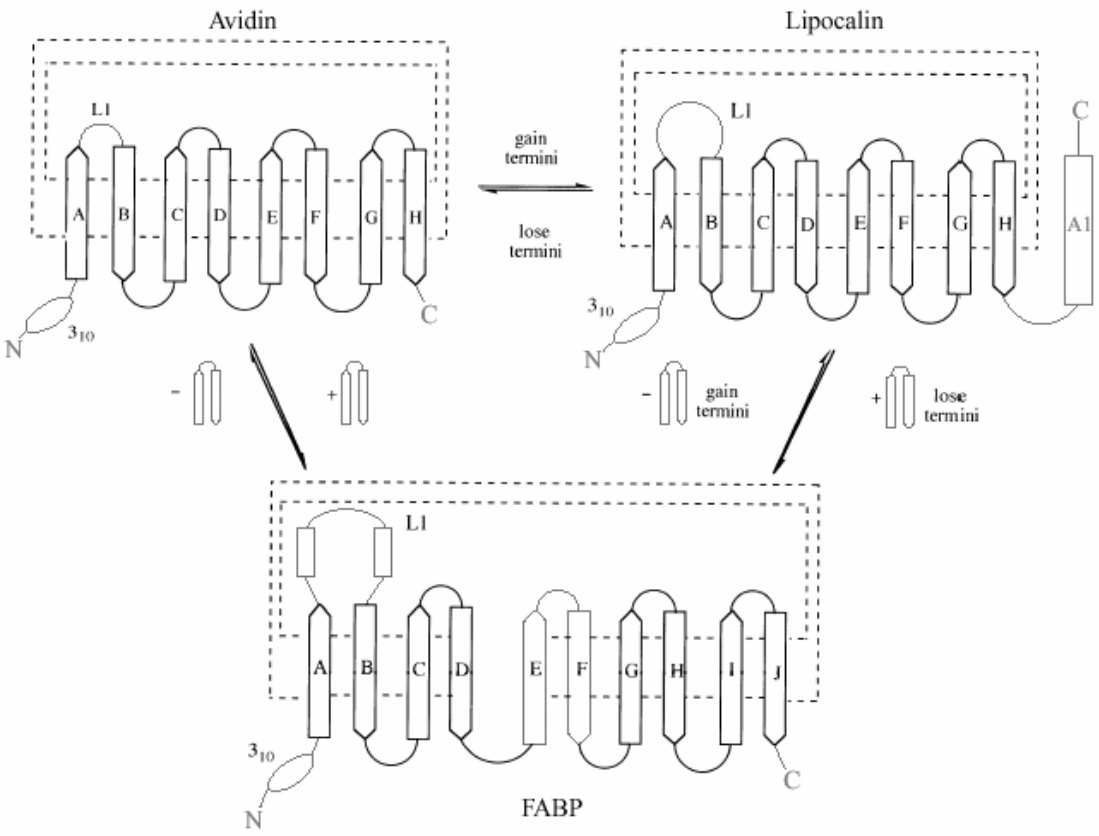

FIGURE 7 Relationship between avidin, lipocalin and FABP. When compared to lipocalin, avidin and FABP lack both a N-terminal sequence stretch (not shown) prior to the 3_{10}-helix and a C-terminal α-helix, labelled A1. Furthermore, FABP contains two additional β-strands when compared to avidin or lipocalin. The loop labelled L1 is also truncated in avidin, when compared to the others. The hydrogen bonds between the β-strands are indicated by dotted lines. The picture was reconstructed from Flower (1996).

The 3D-structures of streptavidin (Hendrickson et al. 1989, Weber et al. 1989), retinol binding protein (RBP; a representative lipocalin) (Cowan et al. 1990) and rat intestinal fatty acid-binding protein (I-FABP; a representative FABP) (Sacchettini et al. 1989) have been superimposed, and the initial four and final two strands were found to be structurally relatively well conserved (Flower 1993). Furthermore, calycins are functionally similar, in that they all bind

hydrophobic ligands. The ligand-binding site is also similarly located inside the β-barrels, which share, as mentioned, similar folding patterns and large equivalent structural parts. Calycins do not share noticeable global sequence similarity, but two intriguing features can be pointed out: a conserved 3_{10}-like helix motif and a peculiar structural signature (Flower 1996). A 3_{10} -helix differs from an ordinary α-helix in that the hydrogen bonds are from the main chain oxygen(i) to nitrogen(i+3), and not from oxygen(i) to nitrogen(i+4). This means that, there are three amino acid residues per turn and the hydrogen bond forms a characteristic cycle of ten atoms, including the hydrogen itself. In calycins the 3_{10} –helix motif is conserved conformationally and spatially. In all known calycin structures it leads always to the first strand of the β-barrel (Fig. 7). This short N-terminal part is the only region which displays significant sequence similarity between the known sequences. The structural signature comprises an arginine or a lysine in the last strand of the β-barrel which packs across a conserved tryptophan from the first β-strand and forms hydrogen bonds with the main-chain carbonyl groups of the N-terminal 3_{10} –helix motif.

From the 3D-structures of avidin (Livnah et al. 1993a, Pugliese et al. 1993, Pugliese et al. 1994) and streptavidin (Hendrickson et al. 1989, Weber et al. 1989) the conserved residues involved in forming the structural signature can be described as follows. The last strand arginine or lysine that packs across a conserved tryptophan is arginine 122 in avidin and lysine 132 in streptavidin, whereas the tryptophan itself is in avidin W10 and in streptavidin W21. Interestingly, the multiple sequence alignment of avidin and the AVRs (Laitinen et al. 2002) mentioned previously shows that the first ten amino acids are identical, with the exception of K9E substitution in AVRs two, six and seven, including the significant tryptophan 10. In addition, arginine 122 is conserved in all the AVRs. Furthermore, all known streptavidins are also identical in regions corresponding to the structural signature, and even the distant Sea urchin avidin-like domains seem to bear this conserved identification mark. These facts together suggest that the calycin structural signature applies to avidin-related proteins, different streptavidins and fibropellin avidin-like domains as well, although their 3D-structures remain to be solved.

2.1.3.5 Sea urchin fibropellins

Sea urchin embryos are surrounded by glycoproteins that form a specific fibrous belt-like structure. Due to this morphological feature the respective proteins were given the name fibropellins (Bisgrove et al. 1991, Bisgrove & Raff 1993). Fibropellins belong to the epidermal growth factor (EGF) gene family, in which they form an exception since they bear multiple EGF-like repeats. This property is rare and usually connected to a role in embryo development (Delgadillo-Reynoso et al. 1989). The N-terminal part of a putative fibropellin protein comprise a signal peptide followed by an EGF-like domain and a domain resembling a domain in the human complement protein, C1s. The

central part is composed of several EGF-like repeats, the exact number of which varies between different fibropellins. Finally, in the C-terminus is a domain that bears significant sequence similarity with avidin and streptavidin (Hursh et al. 1987, Delgadillo-Reynoso et al. 1989, Hunt & Barker 1989). Neither the 3D-structures nor the biotin-binding properties of full-length fibropellins have been determined, but it has been suggested that they could bind biotin, since several putative biotin-binding residues seem to be conserved in the avidin-like domains, which are 129 amino acids long (Hunt & Barker 1989). However, all of the known avidin-like domains have couple unorthodox substitutions among the supposed biotin-binding residues, in particular tryptophan 110 to lysine and tryptophan 70 to arginine, which could have a serious effect on their binding properties. Furthermore, preliminary results with a recombinant avidin-like domain produced in insect cells indicated that the domain is actually devoid of a biotin-binding capability (Nordlund 1999).

The biological function of fibropellin is most probably related to the formation of a fibrous extra cellular matrix together with some other proteins, which forms the basis for cell adhesion, signalling and movement in the different stages of embryo development (Bisgrove et al. 1991, Bisgrove & Raff 1993). All of the domains in fibropellins are supposed to be capable of forming protein-protein interactions, which may better account for the biological function of the avidin-like domain than its ability to bind biotin.

2.2 Modified (strept)avidins

Avidin and streptavidin have been modified chemically and genetically in many ways over a long period of time. The early chemical modification experiments with avidin (Fraenkel-Conrat et al. 1952, Green 1975 and references therein) were done well before the 3D-structure was resolved. These experiments gave valuable information about avidin's structure and function. However, the results obtained were not comprehensive and left some questions open. Over a decade later more detailed results on the importance of lysine (Gitlin et al. 1987), tryptophan (Gitlin et al. 1988) and tyrosine residues (Gitlin et al. 1990) in the binding site were obtained using amino-acid specific reagents.

The high pI of avidin is thought to be a problem in some applications, since it may cause non-specific binding. In order to reduce the charge of avidin, it has been modified chemically by formylation (Guesdon et al. 1979), acetylation (Kaplan et al. 1983) and succinylation (Finn et al. 1984). Nowadays modification of (strept)avidin is mainly performed genetically. The most evident driving force behind this protein engineering is the need of diverse biotin-binding proteins in special applications (Wilchek & Bayer 1990, Wilchek & Bayer 1999). In addition, these studies together with the previous chemical modification have produced valuable information about proteins, protein-protein and protein-ligand interactions.

2.2.1 Binding-site mutants

The biotin-binding site of (strept)avidin has been one of the major targets of site-directed mutagenesis. The wild-type binding site displays a virtually perfect fit with biotin, and therefore mutants with reduced affinity are expected upon the introduction of any mutations to the area (Wilchek & Bayer 1999). Although most of the applications of avidin-biotin technology are based on the extreme affinity between (strept)avidin and biotin, mutants with reduced affinity and reversible binding characteristics would be particularly useful in separation techniques.

Mutant streptavidins in which tryptophans 79, 108 and 120 were substituted separately with alanine or phenylalanine showed altered biotin and 2-iminobiotin binding properties (Chilkoti et al. 1995a). The conservative phenylalanine mutants W79F and W108F showed slightly reduced affinity toward 2-iminobiotin, whereas the affinity of the W120F mutant was estimated to be two orders of magnitude weaker than that of wild-type streptavidin. All the alanine mutants showed severely reduced 2-iminobiotin affinity values, which had decreased by four to six orders of magnitude. Estimated biotin K_d values were 4.3×10^{-7} M (W79A), 8.6×10^{-6} M (W120A) and $>5 \times 10^{-9}$ M (W120F), whereas W108A was unstable in the assay conditions and the assay could not differentiate such high affinities as those displayed by W79F and W108F from that of wild-type streptavidin. In an independent study the biotin K_d value of a W120F mutant was estimated to be $1\text{-}3 \times 10^{-8}$ M and the binding was found to be reversible, since bound biotin was released by the addition of excess free biotin (Sano & Cantor 1995). In addition, they reported that the mutant had weaker subunit interactions than that of wild-type streptavidin both in the presence and in the absence of biotin.

Those residues in streptavidin that form the hydrogen bonds with biotin ureido oxygen have been converted to alanine (N23A, S27A and Y43A), phenylalanine (Y43F) or glutamate (N23E), and the reported affinities for biotin were 282, 114, 67, 6.9 and 69 times weaker than that of wild-type streptavidin (Klumb et al. 1998). In a similar approach with avidin, Y33 (equivalent to the Y43 of streptavidin) was mutated to alanine, phenylalanine, glutamine or histidine (Marttila et al. 2003). The results were in line with those reported for the analogous streptavidin mutants, and the phenylalanine and histidine mutants displayed only a 4 – and 6 – fold decrease in 2-iminobiotin affinity, respectively. The alanine mutant had 50 times weaker affinity toward 2-iminobiotin, and the glutamine mutant had such poor affinity that it could not be measured reliably. Interestingly, all the avidin mutants, except Y33F, also showed markedly reduced stability of the tetrameric quaternary structure when compared to that of wild-type avidin, and the proteins were mainly monomeric in relatively low temperatures. However, biotin binding restored the tetramers in assay conditions but the stability of these biotin complexes was also impaired compared to that of wild-type avidin.

Aspartate 128 in streptavidin has been converted to alanine (Freitag et al. 1999). In wild-type streptavidin this aspartate side-chain carboxylate oxygen is

hydrogen bonded to an ureido nitrogen atom of biotin. In addition, this oxygen is also involved in hydrogen bonds with side-chain nitrogen atoms of W92 and N23. Furthermore, the other carboxylate oxygen is hydrogen bonded to side-chain nitrogen atoms of W108 and Q24. Upon the conversion of aspartate 128 to alanine this network of interactions is broken. The 3D-structure of the mutant indicated that the hydrogen bond between N23 and the biotin ureido oxygen was lengthened to 3.8 Å, and that a water molecule had entered the binding site to replace the missing interactions. It was also stated that the structure closely parallels a key intermediate observed in a simulated dissociation pathway of biotin from streptavidin.

Biotin-binding residues serine 45, threonine 90 and aspartate 128 of streptavidin have been converted separately and in combinations of two and all three to alanines (Qureshi et al. 2001, Qureshi & Wong 2002). These mutants showed a wide spectrum of affinities toward biotin (K_d between 10^{-6} – 10^{-11} M). It was also reported that the mutants were partially or completely monomeric in a SDS-PAGE assay, and that the presence of biotin restored the tetramers of some mutants, although only partially. These results indicated that modification of only biotin-binding residues can have a far-spreading effect on monomer-monomer interactions. However, in another independent study the S45A streptavidin mutant displayed three orders of magnitude lower affinity toward biotin (Hyre et al. 2000) and an almost unaltered quaternary 3D-structure compared to that of wild-type streptavidin. This is somewhat contradictory to the above described results. The D128A mutant showed even more diverse results, since it was described as completely tetrameric on the basis of its 3D-structure (Freitag et al. 1999, previous chapter) and as mainly monomeric in another study (Qureshi et al. 2001). These differences in results can not be explained exhaustively, but the different production and purification (secreted / inclusion body) methods as well as different analytical approaches (biosensor / calorimetry, SDS-PAGE assay / x-ray crystallography) may explain some of them.

In addition to all above mentioned binding-site mutants, based on site-directed amino acid substitutions, an intriguing loop deletion *via* circular permutation of streptavidin has been described (Chu et al. 1998). The objective of the study was to determine the importance of the flexible loop between β-strands three and four that undergoes an open-to-closed conformational change upon biotin binding. In order to do that, the original termini were joined by a tetrapeptide linker and the new N- and C- termini were introduced at glutamate 51 and alanine 46, respectively. As a result the backbone topology was changed dramatically and residues 47-50 including asparagine 49, which in wild-type streptavidin forms a main-chain amide nitrogen to biotin valeryl moiety carboxylate hydrogen bond (Hendrickson et al. 1989, Weber et al. 1989), were deleted. Despite the modification, this circularly permuted streptavidin exhibited well conserved tertiary and quaternary structures but a severely reduced affinity toward biotin (K_d approximately 10^{-7} M).

The biotin-binding site of streptavidin has also been engineered so as to alter the ligand specificity to favour 2-iminobiotin over biotin. This was accomplished by lowering the biotin affinity while retaining the 2-iminobiotin affinity virtually unaltered (Reznik et al. 1998). In that study two of the three amino acid residues, namely N23 and S27, that form hydrogen bonds with the ureido oxygen of biotin, were converted to alanine and aspartate or glutamate, respectively. The biotin affinity of the N23A, S27D mutant collapsed from the wild-type affinity by eight orders of magnitude to a K_d value around 1×10^{-4} M, while the 2-iminobiotin affinity was relatively well conserved and two orders of magnitude higher than that of biotin.

The streptavidin binding site has moderate affinity toward a nine amino acid peptide (Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly) called Strep-tag (Schmidt & Skerra 1993, Skerra & Schmidt 1999), which was obtained from a pool of random peptides displayed as C-terminal fusions on a V_H domain of a F_V fragment of an antibody. The Strep-tag is readily released from streptavidin by the addition of excess biotin or biotin analogues, and only the histidine and glutamine residues occupy part of the space taken up by biotin in the binding site. In addition, a conformational change was observed in the 3D structure of the streptavidin strep-tag complex (Schmidt et al. 1996) involving opening of the loop between β-strands three and four. Strep-tag suffered from the restriction of being fully functional only as a C-terminal fusion. Therefore the sequence was further modified and optimised, and a terminus independent peptide, Strep-tagII was obtained. This tag, however, displayed lower affinity toward streptavidin than the original Strep-tag and therefore streptavidin itself was subjected to modification in order to increase the affinity. For this purpose the residues 44-47 (Glu-Ser-Ala-Val) in the loop between β-strands three and four were subjected to random mutagenesis (Voss & Skerra 1997). Two of the screened streptavidin mutants showed improved affinity of more than one order of magnitude (K_d around 1×10^{-6} M) toward Strep-tagII and the mutant bearing the residues Val-Thr-Ala-Arg (44-47) also showed enhanced performance in the purification of fusion proteins containing Strep-tagII when coupled to a chromatographic support.

2.2.2 Interface mutants

The fact that (strept)avidin is composed of four symmetrically oriented identical subunits is fascinating. Contact patterns between the subunits are well defined and therefore attempts have been made to produce a modified monomeric and dimeric form by breaking certain interactions in the tetramer.

The one-to-three and one-to-four monomer-monomer interactions of avidin have been weakened by mutating the one-to-three interface residues (M96, V115 and I117) and two key-residues (N54 and N69) of the one-to-four interface to alanines (Laitinen et al. 2001) The first set was designed to reduce the hydrophobic nature of the one-to-three interface, whereas the second eliminated some hydrogen-bonding potential from the one-to-four interface. A

series of four mutants was described, all of which showed reduced quaternary structure stability, particularly in the absence of biotin. Two of the mutants bearing the one-to-three alanine substitutions with N54A or with both N54A and N69A were completely monomeric, even at room temperature in a SDS-PAGE assay, and also in gel filtration FPLC in the absence of biotin. However, the presence of biotin unexpectedly induced tetramerization of the mutants, which were almost as stable as wild-type avidin tetramers.

On the basis of the sequence information obtained from the fibropellin avidin-like domain, the functional one-to-two interface residues tryptophan 110 in avidin and 120 in streptavidin have been converted to lysines (Laitinen et al. 1999). The affinity of the resultant avidin (W110K) and streptavidin (W120K) mutant toward biotin collapsed by several orders of magnitude (K_d around 1 x 10^{-8} M), and the nature of the binding was found to be reversible, in the sense that the free excess biotin released most of the bound mutant from the biotin surface. In addition, the mutants were shown to exhibit stable dimeric quaternary structures in solution. The 3D-structures of these dimeric (strept)avidins have recently been resolved (Pazy et al. 2003a). Interestingly, they appeared to be tetramers almost identical to the wild-type proteins in the crystals, indicating a concentration dependent dimer-tetramer transition.

In another study histidine 127, which faces histidine 127 from the neighbouring subunit, was converted to aspartate in order to create electrostatic repulsion between subunits one and three (Sano et al. 1997). This was supposed to prevent the formation of the wild-type tetramer and result in a dimeric form of the protein. However, the mutant formed non-functional aggregates. In order to reduce the hydrophobicity of the mutant it was further modified by deleting part of the loop between β-strands seven and eight containing the amino acid residues G113-W120. Owing to this a β-turn (S112-K121) connecting β-strands seven and eight was supposed to form. This H127D with the loop deletion mutant was shown to be dimeric, but it refolded only in the presence of excess biotin and degraded into non-functional monomers in the absence of the ligand.

Histidine 127 of streptavidin has also been converted to cysteine and lysine in addition to aspartate, as mentioned above. The cysteine mutant was shown to form intermonomeric disulfide bridges (Chilkoti et al. 1995b) as well as irreversible chemically induced covalent bonds (Reznik et al. 1996), and to have in both cases enhanced stability, according to a SDS-PAGE assay after heat treatment and a biotin binding assay in guanidine hydrochloride. The lysine mutant was designed to form hybrid tetrameric streptavidins with the aspartate mutant, in which the lysine ε-amino group from one subunit would form a favourable electrostatic interaction with the β-carboxyl group of aspartate from the neighbouring subunit. In addition, the lysine and aspartate side chains were chemically croslinked together and the mutant was reported to be more stable than wild-type streptavidin.

2.2.3 Other modifications

The β-barrel structure of streptavidin has been used as a scaffold to display cell adhesive hexapeptide sequences derived from osteopontin and fibronectin, which contain the RGD cell adhesion domain (McDevitt et al. 1999, Le Trong et al. 2003). In their initial construct the ATD sequence of streptavidin (amino acids 65-67) in the loop between β-strands four and five was converted to RGD by site-directed mutagenesis. This construct did not, however, promote cell adhesion in an experiment *in vitro* with rat aortic endothelial and human melanoma cells, possibly due to inadequate exposure or a sterically unfavourable structure of the domain. Because of this, they also introduced the RGD flanking regions present in osteopontin and fibronectin into the constructs, and the final sequences, that promoted integrin dependent cell adhesion, were GRGDSP and GRGDSV, respectively. Wild-type streptavidin contains a RYDS sequence stretch (amino acids 59-62) homologous to the fibronectin RGDS. In the above-described study (McDevitt et al. 1999) wild-type streptavidin did not show adhesion to the melanoma and endothelial cells used, whereas in other studies Chinese hamster ovary cells, M4 murine melanoma cells, ADP-activated platelets and CD4+ lymphocytes have shown integrin mediated cell adhesion, possibly *via* this homologous stretch (Alon et al. 1990, Alon et al. 1992, Alon et al. 1993). Furthermore, a large proportion of *in vivo* administered streptavidin is taken up by the kidney, which is a disadvantageous property in several medical applications (Schechter et al 1995, Schechter et al. 1996). It has been supposed that the *in vivo* kidney uptake is integrin-mediated and dependent on the wild-type RYDS domain. This is supported by the results obtained in an experiment, in which the RYDS was mutated to RYES and the resultant point-mutated D61E streptavidin showed markedly reduced cell attachment *in vitro* (Murray et al. 2002).

Reduced antibody response to streptavidin mutants has been described in a site-directed mutagenesis study (Meyer et al. 2001). The objective of the study was to create a less antigenic but tetrameric and strongly biotin-binding form of the protein, which could be administered repeatedly *in vivo*. They hypothesised that the physical forces that stabilise antigen-antibody interactions might also be important in antigen recognition by the immune system. Therefore they assumed that if the epitope residues were suitably replaced, the resulting protein would be less immunoreactive and also bear less antigenicity. They constructed a series of 37 mutants, in which a collection of surface-exposed aromatic, large hydrophobic and charged residues were mainly substituted with serine, glysine or alanine residues. The remaining conversions were more or less conservative, such as E to Q, D to N and K to M. One of the mutants showed less than 10% immunoreactivity compared to that of wild-type streptavidin, but its antigenicity was not reduced and it elicited a strong immune response in rabbits. Another mutant was described to be only 20% as antigenic as streptavidin. In addition, it showed a loss of cross reactivity and

rabbits immunised with it or streptavidin failed to recognise the alternative antigen.

In order to expand the usefulness of avidin in applications, in which its charge-related non-specific binding is detrimental, a series of charge-reduced avidin mutants, with pIs ranging from 9.4 to 4.7, has been described (Marttila et al. 1998). The series contains six different mutants, which have two to six from positive to neutral or negative amino acid substitutions. The mutations were designed using the evolutionary approach, and the R2A and K3E substitutions were designed on the basis of analogous residues present in streptavidin, according to a sequence alignment. Furthermore, the K9E, R26N and R59A substitutions were designed using the sequence information of the putative avidin-related proteins in a similar manner and the R122A and R124A substitutions were designed on the basis of an inspection of the 3D-structure (Livnah et al. 1993a). In parenthesis, the mutation K9D (and not the designed K9E) present in the pI7.2 mutant originated in a PCR error. All the mutants displayed an affinity toward 2-iminobiotin almost as high as that of wild-type avidin, indicating that the substituted surface-residues did not have any major or far-reaching effect on the biotin-binding site. In addition to that, all of the mutants displayed considerably stable quaternary structures, which suggested that also the monomer-monomer interactions remained quite intact despite the multiple modifications. These findings are supported by an independent study, in which a similar acidic (pI 5.5) avidin, with four substitutions was crystallised and the 3D-structure revealed that the inversion of the exposed electrostatic charges had no significant effect on the overall structure (Nardone et al. 1998). Moreover, the pI 4.7 avidin mutant has been further modified by an asparagine 17 to isoleucine substitution. Due to this AVR-derived mutation the N-glycosylation recognition site of avidin was abolished and the resultant non-glycosylated mutant showed excellent non-specific binding properties (Marttila et al. 2000).

A combinatorial alanine scanning method called shotgun scanning (Weiss et al. 2000) has been used to analyse the functional contribution of the 38 C-terminal residues of streptavidin (Avrantinis et al. 2002). In this method, the studied amino acid side-chains are preferentially allowed to vary only between the wild-type and alanine. The phage-displayed mutant streptavidin pool was subjected to biotin-binding selection and the biologically active forms were selected and sequenced. For each amino acid position studied the wild-type / alanine ratio was determined and when a large ratio was observed, i.e. the wild-type side chain was markedly preferred over the alanine substitution in functional streptavidin mutants, the side chain was supposed to have a central role in the biological activity. The phage-display construct was designed to produce tetrameric streptavidin and, therefore both streptavidin subunits and streptavidin phage coat protein P8 fusions were expressed from the same construct. This was accomplished by using an amber (the stop codon UAG) suppressor *E. coli* strain and an amber stop codon between streptavidin and the phage coat protein P8 coding regions (Sidhu et al. 2000). The shotgun scanning results for streptavidin were largely in line with the previous results obtained

from single-point site-directed mutagenesis studies on biotin contact residues. Additionally, new residues with long-distance impact for biotin binding or stability of the tertiary and quaternary structures were resolved.

As mentioned in previous sections, avidin displays pseudo-catalytic activity concerning biotinyl p-nitrophenyl ester alkaline hydrolysis (Huberman et al. 2001), whereas streptavidin is devoid of it, and on the contrary protects the substrate from hydrolysis. The structural features of avidin that are thought to be responsible for this activity have been successfully transferred into genetically engineered streptavidin mutants (Pazy et al. 2003b). In the latter study three mutants were constructed. Mutant M1 carries a single point mutation (Leu 124 to Arg) analogous to the arginine 114 present in avidin, which presumably repels the BNP substrate into a conformation that favors its hydrogen-bond interaction with lysine 111 from the adjacent monomer, which is equivalent to lysine 121 in streptavidin. Mutant M2 contains a loop exchange modification, in which residues 48-52 (GNAES) in streptavidin are substituted with residues 38-45 (TATSNEIK) of avidin. This longer loop of three amino acid residues presumably allows the BNP ester group to be more solvent-accessible and thus more prone to alkaline hydrolysis. Finally, the third mutant M3 is a combination of the mutants M1 and M2. The M1 mutant showed significantly improved hydrolytic activity only above a pH value of 10.5, whereas the M2 mutant was markedly active already at pH 8 and above that as active as avidin. The combined mutant M3 was even more active and at some pH values even showed activity superior to that of avidin.

In order to enhance the performance of streptavidin-based solid-phase assays, a hexapeptide containing a single cysteine residue has been fused to the carboxyl terminus of streptavidin (Reznik et al. 2001). The readily reactive sulfhydryl group of the cysteine was used to immobilise the streptavidin variant on maleimide-coated solid surfaces. The used hexapeptide sequence contained a short four-residue linker (Gly-Gly-Ser-Gly) followed by the cysteine residue and at the C-terminus a proline, which was assumed to protect the protein from exopeptidases. According to the design only two specific, cystein-mediated, covalent bonds between the mutant and a solid surface were supposed to form. This could leave at least two binding sites per tetramer fully accessible to biotinylated macromolecules, which would be a huge improvement when compared to the capacity obtained with wild-type streptavidin immobilised covalently *via* its amino groups. Furthermore, immobilised wild-type streptavidin shows quite different biotin-binding characteristics when compared to those of streptavidin in solution (Rai 1995). The higher binding capacity of the streptavidin variant was confirmed, and in addition it showed only slightly different biotin-binding characteristics when compared to those of streptavidin in solution.

3 AIM OF THE STUDY

Avidin is one of the most widely utilised proteins in the life sciences. So called avidin-biotin technology includes for example purification, labeling, targeting and diagnostic applications. In order to obtain more versatile bio-tools, we modified avidin by means of protein engineering. In addition to that, we were interested in shedding some light on matters concerning what kind of modifications can be made to avidin without disrupting the biotin-binding activity. More specifically the aims of this study were:

1. To introduce intermonomeric disulfide bridges into the avidin tetramer and to study their influence on the thermal stability of avidin as well as reveal the importance of the wild-type intrachain disulfide bridge.

2. To define the minimal changes in the monomer-monomer interface areas required to obtain biotin-binding monomeric avidin.

3. To create a pseudo-tetrameric dual-chain avidin molecule, in which half of the binding sites can be modified independently of the other half by combining two circularly permuted avidins.

4. To study the effect of introduced monomer-monomer interface histidine residues on the subunit assembly and biotin binding activity at different pH values.

4 SUMMARY OF MATERIALS AND METHODS

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

4.1 Mutagenesis of avidin cDNA and construction of recombinant baculoviruses

Avidin cDNA obtained from the pGEMAV plasmid (Airenne et al. 1994) was used as the primary template for all constructs (I-IV).The first site-directed point mutations were introduced using the megaprimer method (Sarkar & Sommer 1990) and the subsequent PCR-amplified product was subcloned into the pFASTBAC1 plasmid (Bac-To-Bac™Baculovirus Expression System, Life Technologies, Gaithersburg, MD). Further mutations were introduced into the pFASTBAC1-derivatives either by using the megaprimer method and subcloning or by the QuikChange mutation system (Stratagene, La Jolla, CA) (I, II, IV). The sequence reorganisation and loop insertions in the circularly permuted avidins and dual-chain avidin (III) were done using standard PCR techniques and subcloning. Recombinant baculovirus genomes were generated by site-specific transposition in DH10bac *E. coli* cells according to the Bac-To-Bac™instructions. The obtained recombinant viral genomes were purified and transfected into cultured Sf9 insect cells (ATCC CRL-1711), which produced the recombinant viral particles used in the subsequent insect cell infections.

4.2 Production and purification of the mutant avidins

The Sf9 insect cells were grown in SF 900IISFM (Gibco BRL) or HyQ SFX (HyClone, Logan, UT) medium with cell density between 0.5 x 10^6 and 4 x 10^6 cells / ml. The cells were changed into a biotin-free medium derivative before

infection with the recombinant baculoviruses. The infections were allowed to proceed for about 72 hours, after which the cells were collected and stored at –20°C or - 80°C for further treatment. Proteins were purified from the cells by breaking them with a combination of a lysis buffer and sonication. Then the cell extracts were centrifuged and the soluble fraction was subjected to biotin (II, IV) or 2-iminobiotin (I, III, IV) agarose affinity chromatography. As required, the eluted proteins were concentrated and subjected to buffer changes using Centricon YM-3 filters (Millipore, Billerica, MA).

4.3 Characterisation of the mutants

4.3.1 Ligand-binding properties

Rough estimations of the 2-iminobiotin and biotin-binding properties of the mutants were obtained during purification, based on their behaviour. Some of the mutants showed inadequate affinity toward 2-iminobiotin agarose and therefore were purified with biotin agarose affinity chromatography (II, IV). Reversibility of surface immobilised biotin binding was determined for the mutants using an IAsys optical biosensor (I-IV). In addition, affinity toward the biotin was determined for monoavidin with the biosensor (II). Similarly, the affinity of several mutants toward immobilised 2-iminobiotin was determined using the IAsys apparatus (I, III). The Affinity of monoavidin toward biotin in solution was also determined by a PerkinElmer SL55 fluorescence spectrometer based on the quenching of the intrinsic fluorescence of the mutant upon biotin binding (II). Moreover, dual-chain avidin was subjected to a colorimetric HABA assay (Green 1970) in order to determine the number of free and active binding sites per dcAvd molecule (III).

4.3.2 Structure, stability and other properties

Gel filtration chromatography was used to study the oligomerization properties of the mutants (II, III, IV). In the case of the interface histidine mutants (IV) the runs were performed for each mutant in acidic, neutral and basic conditions in order to reveal whether the oligomerization was pH dependent phenomenon. A SDS-PAGE-based assay (Bayer et al. 1996), in which protein samples were acetylated (III, IV) and incubated in the presence of SDS-containing sample buffer at different temperatures for 20 minutes prior to gel electrophoresis (II, III, IV), was used to obtain information about the quaternary structure assembly and the stability of the mutants. Moreover, the samples with intermonomeric disulfide bridges (I) were boiled in a SDS-containing but non-reducing sample buffer before gel electrophoresis in order to study the pairing of the introduced cysteines. Thermally induced denaturation of the mutants (I) was studied with a differential scanning calorimeter in order to reveal the effect of the removal or

the wild-type disulfide bridge and introduction of the new intermonomeric disulfide bridges on the thermal stability characteristics. In addition to that, Avd-ci and Avd-ccci, the most stable mutants (I), and wild-type avidin were heated above their melting temperatures and samples were taken at various time points. After that their remaining biotin-binding activity was analysed on the basis of their ability to bind biotinylated alkaline phosphatase in a microtiter-plate assay. The mutants were subjected to proteinase K treatment (II, III) and samples were taken after incubation in the presence of the proteinase at designated time points. The amount of intact protein in the samples as well as the cleavage pattern was determined in a subsequent SDS-PAGE analysis. The immunoreactivity of monoavidin was studied and compared to that of wild-type avidin in an ELISA assay, in which the proteins were immobilised on ELISA plates and probed with monoclonal and polyclonal primary antibodies elicited against wild-type avidin (II). Fluorescence correlation spectroscopy (FCS) studies were conducted to obtain information about the ligand-binding properties and quaternary structure assemblies of the mutants in solution (IV). These measurements were done, similarly to the gel filtration studies (IV), in acidic, neutral and basic conditions.

4.4 Computer programs

We used EMBOSS (European Molecular Biology Open Software Suite), GCG (Genetic Computer Group, Madison, WI), Insight II (Molecular simulations Inc. San Diego, CA) and WHAT IF (Vriend 1990) packages for sequence analysis, mutation design and graphical illustrations.

5 REVIEW OF THE RESULTS

5.1 Avidin mutants with intermonomeric disulfide bridges and a cysteineless avidin (I)

Three different avidin mutants with intermonomeric disulfide bridges were constructed using site-directed mutagenesis. In these mutants cysteine residues were introduced into appropriate locations on the monomer-monomer intefaces, allowing them to form the disulfide bridges with the designed cysteines from the neighbouring subunits. In the avidin mutant Avd-ci isoleucine 117, which faces the analogous isoleusine 117 of the adjacent monomer in the one-to-three (and identically in the two-to-four interface), was converted to cysteine (I, Fig. 1, Fig. 2A). Due to the tetrameric quaternary structure of avidin, the introduction of one cysteine per monomer in Avd-ci produced two intermonomeric disulfide bridges. The design of the second avidin mutant, Avd-cci, was somewhat different in that the introduced cysteines did not form pairs with the same amino acids on the neighbouring subunit but with other amino acid positions. Two cysteines per monomer were thus introduced. Aspartate 86 to cysteine with isoleucine 106 to cysteine substitutions were expected to form two disulfide bridges in the one-to-four (and identically in the two-to-three) interface. As a result of these mutations four new disulfide bridges in total were introduced into the avidin tetramer and all of which were C86-C106 pairs (I, Fig. 1, Fig. 2A). The third mutant with six new intermonomeric disulfide bridges per tetramer, Avd-ccci, was the combination of the mutants Avd-ci and Avi-cci. Finally, in order to create a cysteineless avidin, the two cysteines (C4 and C83) that form the intramonomeric disulfide bridge were substituted with the same amino acids that streptavidin bears in analogous positions, which are alanine and tyrosine, respectively.

All of the mutants were produced with a baculovirus insect cell system. They were soluble, and their good purification efficiency on 2-iminobiotin affinity chromatography indicated that, despite the mutations, the proteins

folded correctly and the 2-iminobiotin binding-properties were not drastically altered. Biotin binding of the mutants was found to be virtually irreversible and indistinguishable from that of wild-type avidin as the free excess biotin did not release the proteins from the biotin surface in an IAsys optical biosensor assay. In addition, the biosensor was also used to determine the affinities of the mutants toward 2-iminobiotin (I, Table 1). The results confirmed that all of the mutants had considerably high 2-iminobiotin affinity, as was expected on the basis of their good purification efficiencies.

In order to find out whether the introduced cysteine residues formed intermonomeric disulfide bridges in the mutants, they were subjected to a SDS-PAGE analysis. Protein samples were boiled for 15 minutes in the presence of SDS but in the absence of the reducing agent β-mercaptoethanol. This should preserve the disulfide bridges, but denature the proteins prior to the analysis. The results showed that the mutants both had intermonomeric disulfide bridges and exhibited dimeric and tetrameric molecular weights, as was expected given their design (I, Fig. 2).

The thermal stability characteristics of the mutants both in the presence and in the absence of biotin were analysed using differential scanning calorimetry (DSC). The results (I, Table 2, Fig. 4) revealed that Avd-ci was the most stable of the proteins studied and that Avd-ccci was only slightly less stable. The T_m value of Avd-ci was increased by 13.1°C in the absence and by 6.5°C in the presence of biotin compared to the values of wild-type avidin under the same circumstances. Similarly, Avd-ccci showed an increase in T_m of 9.2°C and 3.8°C in the absence and presence of the ligand, respectively. Both of these mutants also showed better performance than wild-type avidin in a microtiter plate assay, in which the binding of biotinylated alkaline phosphatase was monitored after heat treatment of the samples (I, Fig. 3). These results showed clearly that by introducing intermonomeric disulfide bridges the thermal stability of avidin can be improved and these mutants provide more robust tools for applications. On the other hand Avd-cci and the cysteineless mutant Avd-nc were found to be clearly less stable than wild-type avidin in the absence of biotin, whereas in the presence of the ligand they were roughly as stable as the wild-type protein.

5.2 Monomeric avidin mutant (II)

The quaternary structure of homotetrameric avidin is defined by three different types of monomer-monomer interactions. The tetrameric assembly is also necessary for the extremely high affinity of avidin toward biotin, mainly because of tryptophan 110, which participates in the biotin binding of the neighbouring subunit. As described in a recent publication, the conversion of tryptophan 110 to lysine in the one-to-two interface resulted in weakening of the tetrameric assembly. The mutant was described as a dimeric avidin with

reversible biotin binding characteristics (Laitinen et al. 1999). Because the formation of a dimer from a tetramer requires that two of the three interfaces are impaired, it was assumed also that the relatively weak one-to-three interface was broken as a result of the manipulation of W110 in interface one-to-two and that in the resultant dimers only the extensive one-to-four interface was retained intact. In another recent publication, asparagine 54 of avidin was converted to alanine in order to reduce the hydrogen bonding potential in the one-to-four interface (Laitinen et al. 2001). This mutation was also found to lead to destabilisation of the tetramer. On the basis of these findings, we decided to combine these two mutations, and resolve the oligomerization and biotin-binding properties of the N54A, W110 double mutant.

The mutant was produced with a baculovirus insect cell system in soluble form. In addition, it was purified efficiently in a single-step protocol on biotin agarose affinity chromatography. An optical biosensor IAsys was used to analyse the biotin binding characteristic of the mutant. Binding to the biotin surface was found to be completely reversible, because the addition of free excess biotin released the surface bound mutant completely (II, Fig. 2A). The dissociation constant (K_d) of the mutant for the biotin surface determined by IAsys was 1-3 x 10^{-7} M (II, Table 1). The K_d of the mutant for biotin in solution was around 7.6 x 10^{-8} M as determined using a fluorescence spectrometer, by monitoring the quenching of the intrinsic fluorescence of the mutant upon biotin binding (II, Fig. 2C).

The mutant was subjected to a SDS-PAGE analysis, in which samples with or without biotin were preincubated at various temperatures for 20 minutes and then applied to electrophoresis gel without boiling. The behaviour of the mutant was dramatically different from that of wild-type avidin. It migrated as a single band with an apparent molecular weight of a monomer already after preincubation at room temperature in both the presence and absence of biotin. The mutant was also analysed by gel filtration chromatography, and it displayed apparent molecular weights of a monomeric form both in the presence and absence of biotin (II, Fig. 3), whereas wild-type avidin was tetrameric in both cases.

The monomeric avidin mutant also displayed reduced immunoreactivity, since two monoclonal antibodies raised against wild-type avidin failed to recognise it completely, and a polyclonal anti-avidin antibody showed clearly reduced recognition in an ELISA assay (II, Table 2). In addition, monoavidin was also shown to be much more sensitive to proteinase K treatment than wild-type avidin, in both the presence and absence of biotin (II, Fig. 4).

5.3 Pseudo-tetrameric dual-chain avidin (III)

In this study we prepared two different circularly permuted forms of avidin and their fusion. The original N- and C- termini were brought together in the

circularly permuted avidins by insertion of a hexa peptide (GGSGGS) linker between them and new termini were introduced into selected loop regions. In the first circularly permuted avidin, named as cpAvd5→4, the loop between the original β-strands 4 and 5 in the non biotin-binding end of the barrel was chosen as the place for the new termini. Therefore, in cpAvd5→4 the original β-strand five is the most N-terminal element and the peptide ends with a C-terminal segment originating from β-strand four. Analogously, the other circularly permuted avidin, named as cpAvd6→5, has new termini in the loop between β-strands 5 and 6, which is in the biotin-binding end of the barrel. Therefore the sequence begins with the N-terminal part derived from the original β-strand six and ends after the original β-strand five. The reason behind the design of the above-described circularly permuted avidins was that the termini were arranged for the subsequent fusion construct, which contained both of the circularly permuted avidins. In this fusion construct, named as dual-chain avidin (dcAvd), the sequence begins with cpAvd5→4, which is further linked to cpAvd6→5 with a tri peptide (SGG) linker between the subunits (III, Fig. 1).

The two circularly permuted avidins cpAvd5→4 and cpAvd6→5 as well as their fusion dcAvd were produced, using a baculovirus insect cell system, as soluble proteins. All of the proteins were efficiently purified on 2-iminobiotin affinity chromatography in a single step protocol. This showed that despite the linkage of the original N- and C- termini and consequential introduction of new termini into the selected loops the circularly permuted avidins were able to fold and form functional barrel structures like those of wild-type avidin. In addition, the fact that also dcAvd was expressed as a functional protein indicated that the fusion of the circularly permuted subunits with the short linker was successful. All of the mutants showed the expected apparent molecular weights in denaturing SDS-PAGE (III, Fig. 2).

Good purification efficiency indicated that the mutants had high affinity toward 2-iminobiotin. This was confirmed by an IAsys optical biosensor and the determined affinities were almost as high as that of wild-type avidin (III, Table 1). In practice, only cpAvd6→5 showed slightly reduced 2-iminobiotin affinity. In the biotin-binding assay the mutants cpAvd5→4 and dcAvd showed as irreversible biotin-binding ability as that of wild-type avidin, whereas cpAvd6→5 was slightly more reversible. However, the reversibility of cpAvd6→5 was only moderate and more than 80 % of the mutant remained bound on the biotin surface in the assay conditions.

The quaternary structure assemblies of the mutants were studied by gel filtration chromatography (III, Table 2). The samples of cpAvd5→4 and cpAvd6→5 eluted as tetrameric forms, which indicated, together with their ligand-binding properties, that the monomer-monomer interactions were similar to those of wild-type avidin, and that the mutations had not caused any major changes to the quaternary structures. In addition, dcAvd also showed an apparent molecular weight similar to that of the other mutants and wild-type avidin. This indicated that it formed a pseudo-tetrameric structure with a

similar overall quaternary structure as that of wild-type avidin, with the designed exception that only two polypeptides formed the four binding sites. In order to confirm that in dcAvd both of the fusion partners were active in the sense of ligand binding, it was subjected to a colorimetric HABA assay. The results proved that both of the fusion partners were fully functional and that the pseudo-tetrameric dcAvd contained at least 3.3 free biotin binding sites per molecule. The stability of the mutants was studied with a SDS-PAGE assay (III, Table 2) and proteinase K assay (III, Fig. 3). All of the mutants were found to be quite stable in both assays, although less stable than wild-type avidin.

5.4 Introduction of histidine residues into subunit interfaces (IV)

In this study a series of six avidin mutants was constructed, in which selected monomer-monomer interface residues were converted to histidines. In the single mutation-bearing versions the one-to-three interface residues methionine 96, valine 115 and isoleucine 117 were substituted separately with histidines. In addition to the previous mutations, the double mutants also contained a tryptophan 110 to histidine conversion in the one-to-two interface (IV, Table 1, Fig. 1). At low pH values the histidine side chain is positively charged due to protonation. Because a one-to-three interface residue interacts with the same residue from the neighbouring subunit, this could lead to repulsion and consequential changes in the quaternary structure assembly. On the other hand, at higher pH values the histidine side chain is not protonated nor charged, and the effect of the mutation might thus not be as radical as at lower pH values. Moreover, as previously shown, when the important biotin-binding and one-to-two interface residue tryptophan 110 (or tryptophan 120 in streptavidin) was converted to a positively charged lysine the tetrameric quaternary structure was devastated, and the biotin-binding affinity of the mutant collapsed by several orders of magnitude when compared to that of wild-type avidin or streptavidin (Laitinen et al. 1999). Therefore we assumed that the tryptophan 110 to histidine substitution might cause a similar, possibly pH dependent, effect, which would enhance the effects generated by the histidine substitutions in the one-to-three interface.

The thermal stability characteristics of the mutants were evaluated by a SDS-PAGE-based assay. According to the results (IV, Table 1, Fig. 2) Avm(I117H), Avm(M96H) and Avm(I117H,W110H) showed T_r values comparable to that of wild-type avidin in the absence of biotin, which indicated that their quaternary structure assemblies were rather stable in the assay conditions. On the contrary, Avm(V115H), Avm(M96,W110H) and Avm(V115H,W110H) were monomeric already at room temperature in the absence of biotin. In the presence of biotin they formed tetramers, except Avm(V115H,W110H), that were clearly less stable than those of wild-type

avidin. In addition, Avm(I117H,W110H) showed a reduced T_r value in the presence of biotin, but Avm(I117H) and Avm(M96H) were virtually as stable as wild-type avidin.

The quaternary structure assemblies of the mutants were studied by gel filtration HPLC analysis in both the presence and absence of biotin at three different pH values. Wild-type avidin as well as Avm(I117H) and Avm(I117H,W110H) were tetrameric in all assay conditions, but the other mutants showed monomeric, dimeric or tetrameric quaternary structures depending on the pH of the buffer as well as the presence or absence of biotin in the samples (IV, Table 2, Fig. 3). The clearest pH dependent subunit association change was observed with Avm(M96H), as it was completely monomeric at pH 4 in the absence of biotin and completely tetrameric at pH 7.2. Moreover, Avm(M96H,W110H) and Avm(V115H) behaved similarly, although the predominant form was monomeric at all pH in the absence of biotin.

The diffusion times obtained for the mutants in the FCS studies (IV, Table 2) supported the gel filtration HPLC results. When a protein exhibited a tetrameric quaternary structure in the presence of biotin in HPLC, a diffusion time close to that seen with the tetrameric wild-type avidin was also observed in FCS. Similarly, on the basis of HPLC, when a protein was not found to be tetrameric in the presence of biotin, the diffusion time was clearly decreased from that seen with wild-type avidin in FCS as well. Protein-label complex formation was also studied with FCS. The value obtained for the protein-label complex (%) as well as the value for the counts per molecule (Cpm) ratio showed clear pH dependency, as these values increased at higher pHs when compared to those observed at pH 4 with Avm(V115H) and all of the double mutants (IV, Table 2). Moreover, when a protein showed, as was the case with wild-type avidin, Avm(I117H) and Avm(M96H), a near 100 % complex formation and a Cpm ratio close to four already at pH 4 it showed the same at the higher pH values as well.

An IAsys optical biosensor equipped with a biotin surface was used to study the immobilised ligand binding properties of the mutants (IV, Table 3). We observed a clear increase in the biotin surface reversibility with Avm(M96H,W110H) and Avm(I117H,W110H), as their moderate reversibility of around 40 % in the assay performed at pH 9.5 changed into almost total reversibility (around 90 %) when the pH was lowered to 4.

6 DISCUSSION

6.1 Avidin mutants with intermonomeric disulfide bridges and a cysteineless avidin (I)

Our goal was to study whether functional avidin can be produced after removal of the wild-type intramonomeric disulfide bridge or introduction of two, four or six intermonomeric disulfide bridges into the tetrameric protein. In addition, we wanted to find out the effect of these modifications on ligand binding and compare the thermal stability characteristics of the mutants with those of wild-type avidin. As discussed earlier, avidin is an extremely stable protein (Green 1975, Green 1990) and, as shown by DSC analysis, it has clearly higher thermal stability than its bacterial relative streptavidin (Gonzales et al. 1999), which is used in several applications. Therefore, we thought that avidin would serve as a convenient starting point when engineering a possibly even more stable biotin-binding protein that might have particular value in experiments and applications in which enhanced stability would be beneficial.

The cysteineless avidin mutant Avd-nc folded correctly and was produced like the other mutants as a soluble protein that displayed ligand-binding characteristics virtually identical to those of the wild-type protein. This proved that the intrachain disulfide bridge in wild-type avidin is not essential for the proper function of the protein. In addition, the alanine (C4A) and tyrosine (C83Y) substitutions designed according to the streptavidin primary structure probably filled the cavity normally occupied by the two cysteines fairly well, since, in the absence of biotin the T_m value obtained for Avd-nc was even slightly better than that reported for streptavidin (Gonzales et al. 1999), which is a natural cysteineless biotin binding protein. Furthermore, in the presence of biotin Avd-nc was as stable as wild-type avidin, which further supported that the mutations were quite convenient, and the wild-type disulfide bridge seemed to stabilise avidin only in the absence of biotin.

A streptavidin mutant with enhanced stability has previously been described (Reznik et al. 1996) but the production and purification of the mutant

was described to be somewhat laborious and time-consuming. On the basis of the mutational strategy used to create an enhanced streptavidin, in which histidine 127 was converted to cysteine, we decided to change the analogous isoleucine 117 in avidin to cysteine. In contrast to the streptavidin mutant, our avidin mutant Avd-ci was produced as a homogenous (excluding the sugar moiety), fully functional and soluble protein, in which the two intermonomeric disulfide bridges between the introduced cysteines were formed in the insect cells without further treatment. Moreover, Avd-ci and the other avidin mutants were purified efficiently in a single-step protocol on 2-iminobiotin affinity chromatography, without employing the denaturation and renaturation procedures commonly involved in the purification of streptavidin mutants from non-soluble inclusion bodies.

The sea urchin fibropellin avidin-like domain, discussed earlier, was used as a template when designing the avidin mutant Avd-cci. The avidin-like domain contains five cysteines and, based on a sequence alignment with avidin, two of them seemed to be analogous with the cysteines that form the intramonomeric disulfide bridge in avidin. One of the three cysteines left to form intra or inter monomeric pairs appeared to be apart from the others in the avidin-like domain, when it is assumed to fold and assemble like the tetramer of avidin. This indicated that it may not form intratetrameric disulfide bridges in fibropellins, which contain a multiplicity of other domains, with domains similar to itself but presumably with other domains or proteins. The final two cysteine positions, on the other hand, were reasonably close in the 3D structure of avidin in the one-to-four (and identically two-to-three) interface in such a manner that the cysteine equivalent to aspartate 86 in subunit one was in the vicinity of the cysteine equivalent to isoleucine 106 of subunit four and *vice versa*. As seen from the SDS-PAGE results (I, Fig. 2), the Avd-cci sample was, as expected, composed of covalently linked subunit dimers; however the DSC results showed that this mutant was less stable than wild-type avidin, indicating that these disulfide bridges failed to stabilise avidin. The one-to-four interface, in which these bridges appeared in Avd-cci is the largest monomer-monomer interface of avidin. Therefore, some sterical hindrance or small change in the quaternary or tertiary structure of the mutant, which could have resulted from non-optimal disulfide bridges, may have weakened some existing interactions while providing new ones.

The combined disulfide bridge mutant, Avd-ccci, was only slightly less stable than the best mutant, Avd-ci. The results obtained separately with Avd-ci and Avd-cci indicated that the enhanced stability of Avd-ccci most probably originated from the I117C mutation, which also remarkably compensated the destabilising effect of the D86C and I106C mutations. For this reason, Avd-ccci might be an even better candidate than Avd-ci in some applications, especially since all of the subunits are directly or indirectly covalently bound to each other, and it remained a tetramer even in denaturing conditions, as shown by the SDS-PAGE results (I, Fig. 2) It is possible, therefore, that even quite robust conditions can be used to treat immobilised Avd-ccci-containing materials

without a deleterious leakage of Avd-ccci subunits and consequential loss of the biotin-binding capacity.

All of the described avidin mutants displayed good 2-iminobiotin affinity as well as irreversible biotin-binding ability. This was not surprising, since the introduced amino acid substitutions did not involve residues directly responsible for biotin binding. Theoretically, the I106C substitution and the subsequent disulfide bridge formation with the D86C cysteine was the most evident mutation that could have changed the binding properties. The reason for this is that I106 resides in the same loop as the important biotin-binding residue W110, and changes in the loop rigidity could hinder the interaction between the tryptophan and biotin. However, this kind of effect was not observed in the mutants Avd-cci and Avd-ccci, which bear the I106C substitution. Due to their virtually unaltered biotin-binding properties, all of the mutants described in this study are suitable for various purposes in avidin-biotin technology. In addition, the enhanced stability characteristics and covalent subunit interactions of Avd-ci and Avd-ccci can also be utilised in some situations in which wild-type avidin would not be sufficiently durable.

6.2 Monomeric avidin mutant (II)

In this study, the mutational strategy was based on observations described in two recent publications. In the first of these it was found that the tryptophan 110 to lysine (Laitinen et al. 1999) conversion in avidin most probably weakens the one-to-two and one-to-three monomer-monomer interactions, although the mutation involved only a residue on the one-to-two interface, and produces a dimeric (one-to-four dimers) form of avidin with reversible biotin binding properties. In the second the asparagine 54 to alanine conversion (Laitinen et al. 2001), in the one-to-four interface, was also found to weaken the subunit interactions. Moreover, when N54A was combined with mutations that weakened the one to three interface, a monomeric avidin, which interestingly formed tetramers upon biotin binding, was obtained. Our aim therefore was to combine the W110K and N54A mutations in order to affect interfaces one-to-two and one-to-three as well as biotin binding with the first mutation and at the same time also impair the extensive one-to-four interface with the latter mutation. In the combined N54A, W110K mutant all of the monomer-monomer interactions were thus compromised, and biotin-binding interplay *via* tryptophan 110 was diminished.

Subsequently, SDS-PAGE assay and gel filtration chromatography confirmed that the mutational strategy used was successful. The mutant exhibited a stable monomeric quaternary structure in both the presence and absence of biotin, and it was therefore named as monoavidin. These results were further supported by the increased proteinase K sensitivity, as monoavidin was degraded completely in a short period of time, even in the

presence of biotin. Wild-type avidin is cleaved only in the absence of biotin and at much slower rate than monoavidin. In addition, monoavidin was cleaved from multiple sites and the proteinase K treatment produced short peptide fragments, whereas wild-type avidin is cleaved only at the loop between β-strands 3 and 4 (Ellison et al. 1995). This indicates that the protective effect of the neighbouring subunits is indeed absent in monoavidin and the exposed former interface regions and other more accessible parts serve as new cleavage sites. Furthermore, two monoclonal antibodies raised against wild-type avidin failed to recognise monoavidin, and polyclonal anti-avidin antibody showed markedly reduced response in an ELISA assay. This may indicate that some structural epitopes involving amino acids from more than one subunit are destroyed as avidin is monomerized.

The affinity of monoavidin toward biotin (K_d~10^{-7} M) collapsed severely compared to that of wild-type avidin (K_d~10^{-15} M). In wild-type avidin the tetrameric quaternary structure and extreme affinity are linked, because in each of the four biotin-binding sites the neighbouring subunit provides the important biotin-binding residue, W110. In monoavidin this linkage is naturally absent, which is evidently the main cause of the observed moderate affinity. The affinity determined for the monoavidin-biotin interaction is also in good agreement with that reported for the chemically treated immobilized monomeric avidin and biotin (Kohanski & Lane 1990), which further supports our results. Bound biotin can be released from monoavidin with excess free biotin in physiological conditions. This property has potential utility, particularly in affinity separation protocols, in which biotinylated materials would be easily released from immobilized monoavidin by mild treatment. Monoavidin would also be a suitable fusion partner, allowing easy elution of immobilized biotin-bound fusion proteins. In addition, the restrictions related to the strong tetramerization propensity of wild-type avidin as a fusion partner are absent from monoavidin. For this reason, also other than monovalent and relatively small proteins could probably be fused to monoavidin.

6.3 Pseudo-tetrameric dual-chain avidin (III)

The total number of reported circularly permuted (strept)avidins is now three. This includes the streptavidin discussed earlier (Chu et al. 1998), in which the loop between β-strands three and four was used as the place for the new termini, and two of our avidin mutants cpAvd5→4 and cpAvd6→5, in which the loops between β-strands four and five, and five and six, respectively, were used for introduction of the new termini. Interestingly, all of these mutants had the new termini in different loops, and they folded correctly and were active in the sense of biotin binding. This proved once again that (strept)avidin is very suitable target for protein engineering, and that various modifications can be introduced, without destroying the functional barrel-structure.

However, our primary target was not to scan the avidin structure for all the permissible positions for new termini. Instead, we arranged the termini in our two different circularly permuted forms so that they could be fused together with little disturbance to structure and function. This was done using a short monomer-monomer linker-peptide. In the tetrameric avidin molecule the most independent monomer pair is the structural dimer of subunits one and four (and identically two and three), which is characterised by multiple monomer-monomer interactions and a large buried surface area between the monomers (Livnah et al 1993a). With respect to the folding and oligomerization of avidin, these monomers most probably recognise each other first, and after that the tetramer is formed, as dictated by the two other monomer-monomer interactions. Therefore the one and four subunit pair was natural choice for subunit fusion. We studied the 3D-structure of the structural dimer in order to find an appropriate place for the subunit to subunit transition. We observed that β-strand four in subunit one is juxtaposed and antiparallel to β-strand six in the neighbouring subunit four. Therefore, if the C-terminus of subunit one was located after β-strand four, as it is in cpAvd5→4, we would easily be able to link it to subunit four, providing it had the N-terminus before β-strand six, as is the case in cpAvd6→5 (III, Fig. 1).

The biotin and 2-iminobiotin binding properties of cpAvd5→4 were found to be nearly identical with those of wild-type avidin. This was an expected result, because neither the biotin-binding end of the barrel nor biotin-binding residues were involved in the reorganisation of the structure topology. On the other hand, we expected and also observed some changes in the binding properties of cpAvd6→5, as the modifications involved biotin contact residues in the loop between β-strands five and six, in the biotin-binding end of the barrel. The binding properties were, however, relatively well conserved, as the 2-iminobiotin affinity dropped only by one order of magnitude, when compared to the streptavidin mutant (Chu et al. 1998), which showed a decrease in biotin affinity of several orders of magnitude. This could be explained by the different loops and proteins that were modified, but also by the fact that we preserved the loop residues, whereas they were deliberately deleted from the streptavidin mutant in addition to the circular permutation. Moreover, the well conserved binding characteristics of dcAvd may result from is well conserved component derived from cpAvd5→4 and moderately conserved component derived from cpAvd6→5. In addition to that, owing to the fusion with the short linker, two of the free termini per structural dimer are absent, and the artificial linker may provide favourable structural support for important amino acids.

When treated with proteinase K, the new hexa peptide loop introduced between the old N- and C-termini seemed to provide a possible proteinase K cleavage site, as in both the presence and absence of biotin all of the mutants were cleaved more rapidly than wild-type avidin, which is virtually resistant to cleavage in the presence of biotin (Ellison et al. 1995). The quaternary structures of the mutants were rather stable as shown by the SDS-PAGE analysis results

(III, Table 2), but clearly less stable than that of wild-type avidin. This is probably explained by the glycine and serine rich and therefore flexible new loops that connected the old termini, as well as provided the monomer-monomer transition. Additionally, breakage of the peptide backbone and consequent appearance of the new termini could also create destabilising effects due to their charges and loss of structural rigidity.

Dual-chain avidin is the first avidin molecule to be described that has two different kinds of binding sites evenly dispersed at the quaternary structure level. Each and every one of the pseudo-tetramers contains two domains derived from cpAvd5→4 and two derived from cpAvd6→5. Because of the genetic linkage of the former subunits the domains can be further modified separately by means of protein engineering. Therefore, dcAvd can be used for various purposes as a novel protein scaffold, enabling, for example, further development of dual-affinity and dual-ligand molecules as well as the controlled spatial arrangement of these different properties at the monomer level.

Theoretically, on account of the interface symmetry between the two structural dimers that form the pseudo-tetramer, dcAvd can have two different quaternary structure assemblies. In the first one, all of the new termini are on the same face of the pseudo-tetramer and subunit one, derived from cpAvd5→4, has the cpAvd5→4 derived part from the other dcAvd monomer in position two and the cpAvd6→5 derived part from the other dcAvd monomer in position three. In the second possible case, the termini of the two dcAvd monomers are on the opposite faces of the pseudo-tetramer, and identical domains share the one-to-three interface, with the result that the different domains interact *via* the one-to-two interface. At the moment we do not know if these two forms coexist or whether one is favoured over the other for some unknown structural reasons. However, we have worked out two possible ways to lock the monomers into a desired position. The first case described above, in which the termini are assumed to be reasonably close in the 3D-structure, could be accomplished by using a simple fusion strategy in which the C-terminus of one dcAvd monomer would be fused to the N-terminus of the second monomer. Moreover, this strategy, yet to be shown to work in practice, would create a single-chain avidin with four binding sites in one polypeptide. The second case on the other hand might be the predominant form, if a cysteine residue would be introduced into one domain of the dcAvd monomer, thereby forming a disulfide bridge with the identical domain from the other dcAvd monomer over the one-to-three interface. A straight forward procedure would be the I117C substitution, used in Avd-ci and Avd-ccci (I), but further experiments need to be conducted before we know if this is possible.

6.4 Introduction of histidine residues into subunit interfaces (IV)

In this study three amino acids, methionine 96, valine 115 and isoleucine 117, in the one-to-three monomer-monomer interface of avidin were replaced separately with histidines. Additionally, these mutants were further modified by a second, tryptophan 110 to histidine conversion. We were interested to find out, if these modifications would change the structural and/or functional properties of avidin. More importantly, we wanted to investigate whether these properties could be rationally adjusted by modifying the pH of the solution, in which the proteins were dissolved. Wild-type avidin is not sensitive even to quite substantial alterations in solution pH (Green 1975). Therefore, a mutant capable of pH dependent oligomerization and/or biotin binding would be useful, particularly in some applications.

The isoleucine 117 to histidine substitution in avidin had virtually no effect on the studied properties of Avm(I117H). This may structurally be explained by the distance of 7Å between the two I117 α-carbons in the one-to-three interface. Extensive ring stacking might, therefore, occur between the introduced histidine residues. Moreover, it seemed also that the interface environment tolerated the histidines well and the wild-type like contact patterns were not destroyed. Interestingly, the double mutant Avm(I117H,W110H) was also a rather stable tetramer; however, the binding characteristics were pH dependent, as shown by the FCS (IV, Table 2) and IAsys biotin-reversibility (IV, Table 3) results. This pH dependency most probably originated from the W110H substitution, which provided supporting evidence for our assumption that histidine could have a similar but adjustable effect at that position as the previously described lysine in W110K avidin or W120K streptavidin (Laitinen et al. 1999). The effect of the histidine was not as radical as that observed with the lysine, as only the biotin binding and not oligomeric assembly was affected. This may be because histidine is more conservative tryptophan substitute than lysine.

On the other hand, the valine 115 to histidine substitution had a significant impact on the studied properties of the avidin mutant. Avm(V115H) was mainly monomeric in the absence of biotin, as shown by the SDS-PAGE (IV, Table 1) and HPLC (IV, Table 2) results. The double mutant Avm(V115H,W110H) showed that W110H substitution enhanced the effect of the V115H and turned the mutant into non-tetrameric form even in the presence of biotin. The results showed that V115H substitution destabilised the tetrameric quaternary structure at all pH values; this may indicate that there is not enough space for a histidine residue in the interface and that some other interactions present in the wild-type protein are perhaps affected.

Finally, the methionine 96 to histidine substitution had an intriguing effect on the avidin mutant. Avm(M96H) formed quite stable tetramers, as shown by the the SDS-PAGE results (IV, Table 1). However, these tetramers were pH

dependent in the absence of biotin at HPLC (IV, Table 2), as the protein was completely monomeric at pH 4 and solely tetrameric at pH 7.2. Addition of the W110H modification caused Avm(M96H,W110H) to be mainly monomeric, except at higher pH, in the presence of biotin. The explanation for this behaviour might be that the α-carbons of the two M96 residues are more than 11 Å apart from each other in the 3D-structure, and therefore there is no extensive ring stacking between the introduced histidines. In contrast, they may repel each other at acidic pHs due to side chain protonation. In addition, when the side chains are not positively charged they fit to the environment quite well, although not perfectly, as the Avm(M96H) and Avm(M96H,W110H) quaternary structures were slightly weaker in the absence of biotin at pH 11 when compared to that observed at pH 7.2 (IV, Table 2).

In conclusion, it seems that in particular Avm(M96H), Avm(M96H,W110H) and Avm(I117H,W110H) could be useful in some applications. The oligomerization state of Avm(M96H) can be regulated by changing the pH of the solution in the absence of biotin, whereas at a low pH tetramerization can be induced by addition of the ligand. Moreover, biotin was able to induce tetramerization of Avm(M96H,W110H) at pH values 7.2 and 11, whereas at pH 4 it was monomeric even in the presence of the vitamin. This might allow an efficient detachment of bound biotinylated substances from the mutant by mild treatment, when desired. Although Avm(I117H,W110H) was tetrameric in all the pH values used its nearly complete reversibility from the biotin surface at pH 4 might have utility in some situations.

7 CONCLUSIONS

In this study chicken avidin was modified both by introducing point mutations into selected positions in the sequence and by reorganizing the topology of the structural elements in desired ways. In addition, new loops were also designed and inserted into avidin in the latter case. Some of the point mutations were designed using the so-called evolutionary approach, in which observed differences in the sequence comparisons with avidin and related proteins were used as a resource for mutation design. In addition, the rest of the point mutations and the other constructs were designed on the basis of the known 3D structure of avidin as well as based on previous findings obtained with different (strept)avidin mutants. The main conclusions of this study are:

1. The intrachain disulfide bridge present in wild-type avidin is not essential for proper folding or biotin binding, and it can be removed by substituting the cysteine residues with the same amino acids that streptavidin bears in analogous positions.

2. Biologically active avidin, in the sense of biotin binding, can be produced after the introduction of intermonomeric disulfide bridges into the tetramer. These bridges provided covalent monomer-monomer linkages and in some cases enhanced the thermal stability of avidin significantly.

3. The propensity of avidin to form a tetrameric quaternary structure assembly can be abolished by only two crucial point mutations, and the resultant monomeric avidin displayed reversible biotin-binding characteristics.

4. The original N- and C-terminus of avidin can be fused with a hexa peptide linker and the new termini can be introduced into the loop between β-strands four and five or five and six. In addition, the two resultant circularly permuted forms can be fused together with an intermonomeric peptide linker. All of these constructs formed

quaternary structures similar to that of wild-type avidin and showed also high-affinity biotin-binding properties.

5. The introduction of histidine residues into structurally and functionally important sites in avidin changes the behavior of avidin markedly. Moreover, some of the mutants displayed pH dependent oligomerization and ligand binding.

Acknowledgements

This study was carried out at the Department of Biological and Environmental Science, University of Jyväskylä. The study was supported partially by a grant from the Finnish Cultural Foundation.

First of all, I would like to thank my supervisor, Professor Markku Kulomaa for all the support that he has given during these years of research. In particular, I value greatly the fact that I had the freedom to decide how to proceed throughout these studies. I also want especially to thank Kristiina Takkinen and Juha Rouvinen for being excellent reviewers.

I would also like to thank all my co-authors: Olli Laitinen, Sanna Uotila, Vesa Hytönen, Eevaleena Porkka, Thomas Nyholm, Ari Marttila, Kari Airenne, Einari Niskanen, Janne Savolainen, Peter Slotte, Edward Bayer, Oded Livnah and Meir Wilchek for their invaluable contributions. In addition to that, I wish to thank Irene Helkala, Pirjo Käpylä, Eila Korhonen, Jarno Hörhä, Laura Pitkänen, Matti Lemmetti, Marjatta Suhonen and Anna-Liisa Kotiranta for their first-rate assistance. I would also like to mention Piia Karisola, Mervi Ahlroth, Maija Vihinen-Ranta, Mirka Salminen, Marja Tiirola, Hong Wang, Satu Pekkala and Eija Kola who among others were responsible for creating an inspiring and friendly lab-atmosphere.

I am also grateful to my parents Marjatta and Rainer as well as my grandparents, other relatives and friends for being there and giving support and encouragement throughout these years.

My warmest thanks go to my wife Nina and sons Jori, Otto and Nuutti for all their support, love and joy. This work is dedicated to you.

YHTEENVETO (Résumé in Finnish)

Avidiinin toiminnan, oligomerisaation, kestävyyden ja rakenne-topologian muokkaaminen

Avidiini on neljästä samanlaisesta alayksiköstä muodostunut tetrameerinen glykoproteiini, jota esiintyy luontaisesti, pieninä pitoisuuksina, kanan munanvalkuaisessa. Eräs avidiinin merkittävimmistä ominaisuuksista on sen kyky sitoa vitamiini biotiinia. Avidiinin ja biotiinin välinen vuorovaikutus on voimakkain tunnettu luonnossa esiintyvä ei-kovalenttinen sidos proteiinin ja sen ligandin välillä. Tätä ominaisuutta on hyödynnetty jo pitkään lukuisissa bio- ja lääketieteellisissä sovelluksissa, jotka yhdessä muodostavat niin kutsutun (strept)avidiini-biotiini –teknologian. Avidiini on lisäksi poikkeuksellisen kestävä proteiini, ja biotiinin sitoutuminen kussakin alayksikössä sijaitsevaan sitomistaskuun saa aikaan vielä merkittävän kestävyyden lisäyksen. Tästä syystä avidiinia voidaan käyttää melko haasteellisissakin olosuhteissa, kuten korkeissa lämpötiloissa, joissa moni muu proteiini menettäisi biologisen aktiivisuutensa.

Avidiinin aminohapposekvenssi on tunnettu, ja sitä koodaava geeni samoin kuin cDNA:kin on kloonattu. Lisäksi sen kolmiulotteinen rakenne on selvitetty röntgensädekristallografian avulla ja sitä voidaan tuottaa rekombinanttisena proteiinina esimerkiksi baculovirus-hyönteisolu –systeemin avulla. Avidiinia on myös tutkittu pitkään biokemiallisesti, joten sitä ja sen toimintaa koskevaa tietoa on runsaasti saatavilla. Näistä seikoista johtuen muokattujen rekombinanttisten avidiinien suunnitteleminen, tuottaminen, puhdistaminen ja luonnehdinta on mahdollista.

Väitöskirjatyöni päätavoitteena oli muokata avidiinia geenitekniikan suomin keinoin siten, että sen ominaisuudet muuttuisivat paremmiksi joitakin olemassa olevia sovelluksia ajatellen sekä avaisivat aivan uusia mahdollisuuksia kehittää lisää sovelluksia.

Ensimmäisessä osatyössä halusimme selvittää, mikä on avidiinin aminohappoketjun sisäisen rikkisillan merkitys, sekä voidaanko avidiinin lämpökestävyyttä parantaa lisäämällä alayksiköiden väliin uusia rikkisiltoja. Sisäisen rikkisillan poistaminen ei vaikuttanut merkittävästi avidiinin toimintaan, joskin kyseisen mutantin lämpökestävyys heikkeni tämän seurauksena, kun näytteessä ei ollut biotiinia. Onnistuimme parantamaan avidiinin lämpökestävyyttä merkittävästi ennalta suunniteltuihin kohtiin sijoitettujen kysteiinien välille muodostuneiden alayksiköiden välisten rikkisiltojen avulla.

Toisessa osatyössä tarkoituksena oli saada aikaan mahdollisimman pienillä muutoksilla aikaan biotiinia sitomaan kykenevä avidiini, joka ei muodostaisi tetrameeriä vaan olisi monomeerinen. Onnistuimme tässä tekemällä ainoastaan kaksi muutosta aminohappoihin, jotka ovat keskeisessä roolissa alayksiköiden välisissä vuorovaikutuksissa.

Kolmannessa osatyössä teimme edellä kuvattuja pistemutaatioita radikaalimpia muutoksia, koskien aminohapposekvenssin uudelleen järjestelyä siten, että avidiinin alkuperäiset N- ja C-terminukset yhdistettiin toisiinsa ja uudet terminukset laitettiin rakenteessa haluttuihin kohtiin kahdella eri tavalla. Tämän seurauksena rakennetopologia muuttui niin, että pystyimme lopulta yhdistämään nämä kaksi erilaista alayksikköä yhdestä polypeptidistä muodostuvaksi kokonaisuudeksi käyttäen rakenteen kannalta edullista menettelyä. Kehittämämme alayksikköyhdistelmä mahdollistaa jatkossa esimerkiksi sellaisten avidiinien kehittämisen, joissa on erilaisia ligandin sitomisominaisuuksia täsmällisesti jakautuneina kvaternaarirakennetasolla.

Neljännessä osatyössä muutimme keskeisiä alayksiköiden välisiin vuorovaikutuksiin ja biotiinin sitomiseen osallistuvia aminohappoja histidiineiksi, joiden sivuketju kykenee saamaan positiivisen varauksen happamissa olosuhteissa. Tämän seurauksena saimme aikaan muutoksia sekä ligandin sitomisessa että avidiinin kyvyssä muodostaa oligomeerisiä rakenteita. Osassa tapauksista muutokset olivat riippuvaisia liuoksen pH:sta ja luultavasti johtuivat lisäämiemme histidiinien varausten aiheuttamista vuorovaikutusmuutoksista.

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