

Ari Marttila

Engineering of Charge, Biotin-binding and
Oligomerization of Avidin:
New Tools for Avidin-Biotin Technology

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UNIVERSITY OF JYVÄSKYLÄ

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ABSTRACT

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Diss.

Avidin is a tetrameric glycoprotein found in chicken egg white. Due to its remarkably high affinity for biotin, avidin has widely been used in biological and biotechnical applications in life sciences. Avidin is also a highly stable protein, maintaining its functional tetrameric structure at high temperatures, extremes of pH, and in the presence of strong denaturants.

In the present study we wanted to find out whether different properties of avidin can be modified with protein engineering using rational design. We used the 3D-structure of avidin together with sequence information from various avidin-like proteins to plan these changes. Besides shedding more light on the protein chemical bases for the high-affinity binding, stability or the physicochemical characteristics of avidin, our aim was also to create new tools for the avidin-biotin technology.

First we demonstrated, that the charge properties of avidin could be modified without affecting its crucial biotin-binding activity by constructing a series of fully functional avidin mutants with isoelectric points ranging from 9.4 to 4.7. Secondly, we manufactured a non-glycosylated avidin mutant and combined it with one of the charge variants. The resultant mutant still bound strongly to biotin but exhibited substantially reduced non-specific binding characteristics when compared to those of the native avidin. Thirdly, by introducing successive alanine mutations into interface residues of avidin we were able to create two monomeric avidin variants, which upon biotin binding reassembled into functional tetramers. Finally, we also substituted the important Tyr-33 of avidin with various amino acids in order to create more reversible biotin-binding variants suitable for applications like affinity purification or protein immobilization.

Key words: Avidin-biotin technology; biotin-binding protein; protein engineering; rational design.

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CONTENTS

List of original publications.....	7
Responsibilities of Ari Marttila in the articles comprising this thesis.....	8
Abbreviations.....	9
1 INTRODUCTION	11
2 REVIEW OF THE LITERATURE.....	13
2.1 Avidin.....	13
2.1.1 General properties of avidin.....	13
2.1.2 Structural and functional features of avidin.....	15
2.1.2.1 Overall structure of avidin.....	15
2.1.2.2 Biotin-binding site.....	17
2.1.2.3 Molecular basis for the high biotin affinity of avidin.....	18
2.1.3 Avidin-like proteins.....	19
2.1.3.1 Streptavidin.....	19
2.1.3.2 Avidin-related genes and putative AVR-proteins.....	21
2.1.3.3 Fibropellins.....	21
2.2 (Strept)avidin-biotin technology.....	22
2.3 Protein engineering.....	25
2.3.1 Rational design.....	26
2.3.2 Directed evolution.....	28
3 AIM OF THE STUDY.....	31
4 SUMMARY OF MATERIALS AND METHODS.....	33
4.1 Site-directed mutagenesis and construction of recombinant baculoviruses.....	33
4.2 Production and purification of avidin mutants.....	33
4.3 Protein analysis.....	34
4.3.1 Stability assays.....	34
4.3.2 Biotin-binding activity.....	34
4.3.3 Non-specific binding assay.....	35
4.3.4 Preparation of Avm-Y33H column.....	35
4.4 Computer programs utilized.....	36
5 REVIEW OF THE RESULTS.....	37
5.1 Reduced charge avidin mutants (I).....	37
5.2 A non-glycosylated and acidic mutant of chicken avidin (II).....	39

5.3	Two monomeric avidin mutants that undergo biotin-induced tetramerization (III).....	41
5.4	Avidin mutants with reduced biotin-binding capacity (IV).....	42
6	DISCUSSION.....	44
6.1	Avidin mutants with reduced charge (I).....	45
6.2	A non-glycosylated and acidic variant of avidin (II).....	46
6.3	Monomeric avidins that undergo tetramerization upon biotin binding (III).....	49
6.4	Avidin mutants with lowered affinity for biotin (IV).....	51
7	CONCLUSIONS.....	55
	<i>Acknowledgements</i>	57
	YHTEENVETO (Résumé in Finnish).....	58
	REFERENCES.....	60

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following scientific articles, which in the text will be referred to by their Roman numerals. In addition, some unpublished results are presented.

- I Marttila, A. T., Airene, K. J., Laitinen, O. H., Kulik, T., Bayer, E. A., Wilchek, M. & Kulomaa, M. S. 1998. Engineering of chicken avidin: a progressive series of reduced charge mutants. *FEBS Letters* 441: 313-317.
- II Marttila, A. T., Laitinen, O. H., Airene, K. J., Kulik, T., Bayer, E. A., Wilchek, M. & Kulomaa, M. S. 2000. Recombinant NeutraLite avidin: a non-glycosylated, acidic mutant of chicken avidin that exhibits high affinity for biotin and low non-specific binding properties. *FEBS Letters* 467: 31-36.
- III Laitinen, O. H., Marttila, A. T., Airene, K. J., Kulik, T., Livnah, O., Bayer, E. A., Wilchek, M. & Kulomaa, M. S. 2001. Biotin induces tetramerization of a recombinant monomeric avidin: a model for protein-protein interactions. *J. Biol. Chem.* 276: 8219-8224.
- IV Marttila, A. T., Hytönen, V. P., Laitinen, O. H., Bayer, E. A., Wilchek, M. & Kulomaa, M. S. 2002. Mutation of the important Tyr-33 residue of chicken avidin: Functional and structural consequences. Manuscript submitted to *Biochemical Journal*.

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RESPONSIBILITIES OF ARI MARTTILA IN THE ARTICLES COMPRISING THIS THESIS

Articles I and II: I was mainly responsible for the planning, the practical work and the writing of these studies. The biotin-binding analyses were done in part by Olli Laitinen and the stability analyses were mainly done by our Israeli co-workers Professors Meir Wilchek and Edward Bayer.

Article III: Olli Laitinen did the major part of the work in this study. I participated in the design of the mutants, the protein analysis and the writing of the article.

Article IV: I was mainly responsible for the planning, practical work and writing of this study. The stability and FPLC analyses were done in part in the laboratory of Meir Wilchek and Edward Bayer. Vesa Hytönen also participated in the practical work by doing some of the binding and stability analyses.

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

ABBREVIATIONS

<i>Avr</i>	avidin-related genes
AVR	avidin-related proteins
ATP	adenosine triphosphate
BSA	bovine serum albumin
3D	three-dimensional
GFP	green fluorescent protein
EGF	epidermal growth factor
FPLC	fast protein liquid chromatography
HPLC	high pressure liquid chromatography
K_d	dissociation constant
m.o.i	multiplicity of infection
pfu	plaque forming unit
pI	isoelectric point
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
<i>Sf</i>	<i>Spodoptera frugiperda</i>
T_m	midpoint of thermally induced denaturation (melting temperature)
VIPa	vasoactive intestinal peptide analog
v-Src	Rous sarcoma virus tyrosine kinase

1 INTRODUCTION

Chicken egg-white avidin is a tetrameric glycoprotein that binds a small vitamin, biotin, with an affinity that is among the highest displayed for noncovalent interactions between a ligand and a protein (Green 1975). This strong interaction has been utilized over the years in numerous biological and biotechnological applications (reviewed by Wilchek & Bayer 1990). The solution of the three-dimensional structures for both avidin (Livnah et al. 1993, Pugliese et al. 1993, Pugliese et al. 1994) and the related bacterial protein streptavidin (Hendrickson et al. 1989, Weber et al. 1989), with and without biotin, has also provided a tool for the better understanding of the high-affinity protein-ligand interactions in general.

Besides its extraordinary strong biotin-binding ability, avidin also exhibits a number of other interesting features. For example, avidin is an extremely stable protein: its tetrameric structure withstands different denaturants, a wide range of pH and temperature conditions and even protease treatments (Green 1975). The remarkable stability of avidin is further increased in the presence of biotin when temperatures well over 100 °C are required for denaturation of the protein (Gonzales et al. 1999). Furthermore, avidin has an unusually high isoelectric point (pI~10.5) (Green 1975), meaning that it generally has strong positive charge. In regard to the (strept)avidin-biotin technology, the high pI of avidin together with the presence of carbohydrate residues have been a constant hindrance to its use in certain applications, due to non-specific binding to extraneous material. For this reason streptavidin, a non-glycosylated and nearly neutral bacterial counterpart of avidin, has virtually replaced avidin in many applications, even though avidin contains more lysine residues for the potential attachment of probes, is more hydrophilic, and is considerably more abundant and cheaper than streptavidin.

Protein engineering technology involves creating new proteins by modifying existing ones. Methods commonly employed in this field include the

use of structure-directed point mutations, comparative analysis with subsequent directed mutagenesis, region-specific mutagenesis, random mutagenesis and *in vitro* evolution. In combination these techniques provide an impressive arsenal of tools for creating proteins with novel properties. The choice of the design and engineering method depends largely on the type of the information known about the protein and its function.

In the case of avidin, the availability of the crystallographic structure together with the sequence information from streptavidin, avidin-related genes (Keinänen et al. 1988, Keinänen et al. 1994, Ahlroth et al. 2000) and the avidin-like C-terminal domain of sea urchin fibropellins (Hursh et al. 1987, Hunt & Barker 1989, Bisgrove et al. 1991) have made it possible to use site-directed mutagenesis in an intelligent manner. In the present study, we wanted to investigate whether some of the features (high pI, glycosylation, oligomerization, biotin binding) of avidin could be engineered through the guidance of structural relatives, i.e. using kind of evolutionary approach. Therefore we first constructed a series of avidin charge mutants with pIs ranging from 9.4 to 4.7 (I). We then mutated the glycosylation site of avidin to produce a mutant without the oligosaccharide moiety and combined this mutant with one of the charge mutants (II). In addition, we were interested to investigate whether it is possible to break down the tetrameric structure of avidin into functional monomers by substituting some of the amino acids in the interfaces between the different subunits (III). Finally, we also created avidin variants with reduced biotin-binding affinity by replacing the important Tyr-33 residue of avidin with different amino acids (IV).

2 REVIEW OF THE LITERATURE

2.1 Avidin

The avidin story began already a century ago when it was found that raw egg-white, as a sole source of protein in the diet, caused a series of anomalies in test animals (Steinitz 1898, Bateman 1916). A few years later the reason for these toxic effects was shown to be avidin, an egg-white protein, which bound biotin tightly and therefore deprived the animals of this important vitamin (Eakin et al. 1941). Ever since those early days there has been great interest in avidin, mostly due to its remarkable biotin binding capacity and later also in its use as a tool for different applications in the life sciences.

2.1.1 General properties of avidin

Avidin is a minor component (up to 0.05 % of total protein) in the egg white of birds, reptiles and amphibia (Green 1975, Elo 1980, Korpela et al. 1981). In the chicken, avidin is produced in the oviduct under the control of the steroid hormone progesterone (reviewed in Tuohimaa et al. 1989). Besides the oviduct, the production of avidin is also induced in several other tissues in both the male and the female chicken during bacterial or viral infections, inflammation or tissue trauma (Elo et al. 1979a, Elo et al. 1979b, Elo et al. 1980, Korpela et al. 1982). It is most likely that both the progesterone-induced and the inflammation-associated avidins are products of the same gene with multifactorial regulation (Kunnas et al. 1993).

The specific biological function of avidin remains uncertain. The strong affinity of avidin for biotin has suggested that its main role might be in acting as a defense protein; in other words to prevent the growth of certain microbes by excluding the vitamin biotin (Green 1975, Tuohimaa et al. 1989). Recently it has also been proposed that avidin may regulate cell proliferation in chicken

myoblasts and chondrocytes by binding extracellular biotin and thereby interfering with fatty acid biosynthesis during terminal cell differentiation (Zerega et al. 2001). Avidin has also been shown to exhibit pseudocatalytic properties since it enhances the alkaline hydrolysis of certain biotinyl ester derivatives (Huberman et al. 2001).

Avidin is a tetrameric protein that consists of four identical subunits each bearing 128 amino acids (DeLange & Huang 1971). Each of these subunits binds one molecule of biotin with extremely high affinity ($K_d \sim 10^{-15}M$) (Green 1975). Avidin is also basically charged protein (pI~10.5) with each monomer possessing eight arginine and nine lysine residues (DeLange & Huang 1971). In addition, the polypeptide chain of avidin contains a glycosylation site at residue asparagine 17. The carbohydrate moiety accounts for about 10 % of the molecular mass of avidin. It is comprised mainly of mannose and N-acetylglucosamine residues and exhibits extensive glycan microheterogeneity (Bruch & White 1983). According to the amino acid sequence, the molecular weight of the avidin tetramer without the sugar moiety is 57 120 Da. Thus, the total molecular weight of the glycosylated tetramer is about 62 400 Da (Bayer & Wilchek 1994).

Furthermore, avidin is a remarkably thermostable protein: the t_m for the unfolding transition of the protein without biotin is 85 °C. The midpoint temperature of denaturation further increases to 117 °C under saturating biotin conditions thereby representing one of the greatest thermal stabilities known for protein described from mesophilic organisms (Gonzales et al. 1999). The avidin-biotin complex can also withstand strong chemically denaturant conditions such as 8 M urea or 6 M guanidinium hydrochloride over a wide pH range (Green 1975).

The production of recombinant avidin proved to be more difficult than that of its bacterial counterpart streptavidin, which can be purified from *E. coli* in high yields (Sano & Cantor 1990). Early attempts to produce avidin in *E. coli* were not very successful, since only small amounts of soluble and functional protein were obtained (Chandra & Gray 1990, Airene et al. 1994). However, a few years later it was shown that biologically active recombinant avidin can be efficiently produced in the baculovirus expression system (Airene et al. 1997). Subsequently, it has been reported (Nardone et al. 1998) that avidin can also be obtained more efficiently from *E. coli* using a denaturation/renaturation protocol adapted from that for the recombinant streptavidin (Sano & Kantor 1990). Recently recombinant avidin has also been produced in transgenic maize with promising results (Kusnadi et al. 1998). In addition, avidin has been successfully expressed using retroviruses (Walker et al. 1996) and Semliki forest virus expression system (Juuti-Uusitalo et al. 2000).

2.1.2 Structural and functional features of avidin

2.1.2.1 Overall structure of avidin

After many difficulties in growing suitable crystals for diffraction (Green & Joynson 1970, Pinn et al. 1982, Gatti et al. 1984), the three-dimensional structure of avidin, with and without biotin, was finally determined by two groups (Livnah et al. 1993, Pugliese et al. 1993, Pugliese et al. 1994). The size of the functional avidin tetramer is about 56 x 50 x 40 Å and the four identical subunits assemble in a quaternary structure with nearly exact 222 molecular symmetry, which positions the two pairs of the binding sites on opposite sides of the protein.

In the quaternary assembly the four subunits of avidin give rise to three distinct subunit interfaces with different structural properties (Fig. 1). These monomer-monomer interactions are responsible for the rigidity of the tetrameric structure and also for forming part of the framework for the strong binding of biotin. Interaction 1-4 makes the largest contribution to the quaternary structure with excessive monomer-monomer interface (1951 Å² per monomer). Most of the contacts in this interface include hydrophobic interactions (van der Waals forces), but there are also important hydrogen bonds involving polar residues and water molecules. In this respect, the most prominent examples are Asn-54 and Asn-69, which form seven and three hydrogen bonds, respectively, through their side chains.

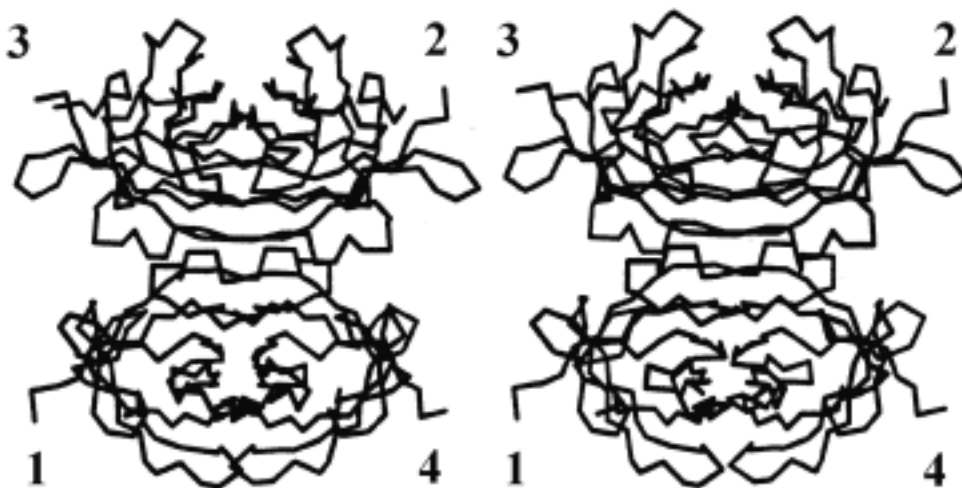


FIGURE 1 Stereo picture of avidin tetramer using C_α-backbone trace. The four monomers are labeled with numbers. The figure was reconstructed from Rosano et al. (1999).

The 1-2 interface also includes several hydrogen bonds and hydrophobic contacts displaying a buried surface area of 729 Å² per monomer. On the other hand, the 1-3 interface is very loose and comprised of only three hydrophobic

amino acids (Met-96, Val-115 and Ile-117). It is characterized solely by van der Waals forces with a surface area of only about 120 Å².

The tertiary structure of the avidin monomer consists of eight antiparallel β -strands (up-and-down topology), which form a classical β -barrel, characterized by a conventional right-handed twist (Fig. 2). The biotin-binding site is positioned near one end of the barrel (pointing upwards in Fig. 2) forming a well-defined cavity, which is oriented approximately along the barrel axis. The loops connecting the antiparallel β -strands vary between 5 and 12 amino acids in length. In the absence of biotin, the electron density of the loop that connects strand β 3 to β 4 (residues from Ala-36 to Asn-42) is not defined due to conformational disorder. However, upon biotin binding, the loop becomes ordered and locks biotin into the binding site.

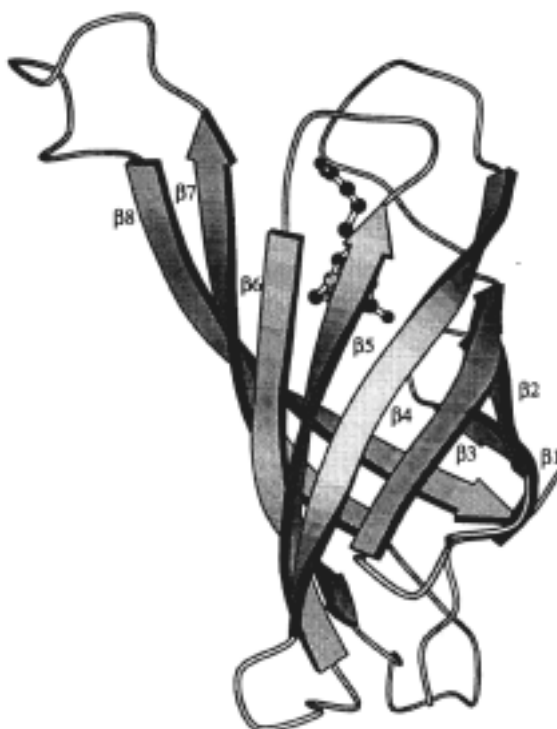


FIGURE 2 A MOLSCRIPT ribbon diagram of the avidin monomer with biotin. The eight strands of the β -barrel are labeled and the biotin molecule is shown in a ball and stick model (Livnah et al. 1993).

In addition to the hydrogen bonds that connect the eight strands of the β -barrel, two more groups of intramolecular hydrogen bonds are found in avidin. The first group (involving residues Ser-5, Leu-6, Trp-10, Gln-61, Ile-85 and Glu-91) establish a polar network that seals the other end of the barrel located opposite the biotin-binding site entrance. This lid is further tightened by a salt bridge between residues Glu-91 and Arg-122 together with the bulky side chain of Trp-10. The hydrogen bonds in the other group connect polar amino acids (Asn-12, Asp-13, Ser-16, Tyr-33, Thr-35, Trp-70 and Thr-77) positioned deep in the biotin-binding pocket and are partly responsible for maintaining the shape of the pocket in the absence of the ligand. Furthermore, there is one intramolecular

disulfide bridge in each avidin monomer, which connects residues Cys-4 and Cys-83 located in the N-terminal region and in β -strand 6, respectively.

2.1.2.2 Biotin-binding site

According to the X-ray crystal structures of avidin (Livnah et al. 1993, Pugliese et al. 1993, Pugliese et al. 1994), the biotin-binding site is a deep pocket with evaluated molecular volume of 293 \AA^3 situated close to one end of the β -barrel. This volume is in good agreement with the molecular volume of biotin, which has been estimated to be around 242 \AA^3 (Rosano et al. 1999). Interestingly, the binding site residues seem to be carefully positioned to provide a precise fit for biotin. In fact, it has been shown that in the absence of biotin, avidin contains five water molecules, which form a defined structure within the binding site. These waters are most likely necessary for maintaining of the shape of the binding site prior to the association with the ligand (Wilchek & Bayer 1999).

The biotin-binding pocket in avidin can be divided into three parts. The deep end of the pocket exhibits a localized cluster of potential residues (Asn-12, Ser-16, Tyr-33, Thr-35 and Asn-118) for hydrogen bonding. These amino acids recognize the polar head of the biotin ureido ring. The middle section of the binding pocket forms kind of an elongated canal lined with hydrophobic amino acids (Leu-14, Trp-70, Phe-79, Leu-99 and Trp-110 from the neighboring subunit). The solvent exposed upper part of the binding site, on the other hand, contains polar residues (Thr-38, Thr-40, Ser-73 and Ser-75) with hydrogen bonding potential.

Bound biotin has little effect on the conformation of the residues lining the binding pocket, despite the numerous specific interactions between them (Fig. 3). In the deep end of the binding pocket the ureido oxygen of biotin molecule forms three hydrogen bonds with the side chains of Asn-12, Ser-16, and Tyr-33 of avidin (Fig. 3B). In addition, each of the two ureido nitrogens are engaged in single hydrogen bond interaction with Thr-35 and Asn-118, respectively. All these five amino acids reside deeply within the protein core and are not accessible to solvent after biotin has been bound. On the other side of the binding pocket, the tetrahydrothiophenic ring of biotin is surrounded by aromatic amino acids Trp-70, Phe-79, Trp-97 and Trp-110 (Fig, 3A). Moreover, residue Phe-72 also further strengthens the structure and the hydrophobicity of this region, although it is not directly in contact with biotin. Of these aromatic residues, Trp-110 is especially interesting, since it is derived from the neighboring subunit. Indeed, the importance of this interaction between the adjacent monomers for the tetrameric structure and biotin binding of avidin has recently been demonstrated in a study where this Trp-residue was replaced with Lys. This unconventional mutation resulted in a dimeric protein with diminished biotin-binding ability (Laitinen et al. 1999).

In addition to the interactions displayed by the bicyclic ring of biotin, the two carboxylate oxygens of the aliphatic valeryl moiety also form hydrogen bonds with avidin (Fig 3B). One of the oxygens interacts with the main chain N-H of Ala-39 and Thr-40 as well as with the side chain of Thr-38, whereas the other forms hydrogen bonds with the side chains of Ser-73 and Ser-75. The aliphatic chain of biotin resides in the elongated channel-like part of the binding pocket that is defined by the closure of the loop 3-4 upon ligand binding. In fact, three of the hydrogen bond interactions formed by the carboxylate oxygens result from this structural change, since this loop contains amino acids 35-46 of avidin.

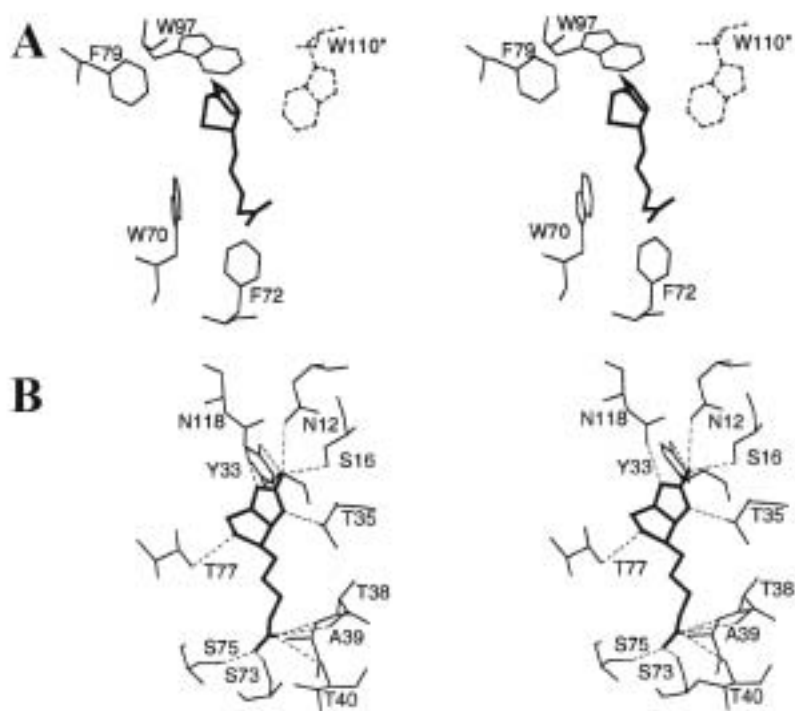


FIGURE 3 Biotin-binding site of avidin. (A) Hydrophobic residues of the biotin-binding pocket: Trp-70, Phe-72, Phe-79, and Trp-97 from one monomer and Trp110 (dashed line), which is provided by the adjoining symmetry-related monomer. (B) Hydrogen bonding interactions between avidin and biotin. For details, see text. The figure was reconstructed from Livnah et al. (1993).

2.1.2.3 Molecular basis for the high biotin affinity of avidin

The remarkably high affinity of biotin towards avidin stems from a high association rate constant ($7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) and from extremely slow dissociation kinetics ($k_{\text{off}} 4 \times 10^{-8} \text{ s}^{-1}$) (Green 1990). The fast binding of biotin means that the activation energy for the association must be low. This is reflected in the 3D-structure of avidin (Livnah et al. 1993, Pugliese et al. 1993, Pugliese et al. 1994), since the binding pocket, without biotin, is fairly open. This is mostly due to the 3-4 loop, which, in the absence of the ligand, is not ordered and therefore allows

easy access to the binding site. Furthermore, as previously discussed, the binding pocket appears to be precisely designed to fit biotin and therefore no conformational changes requiring energy are needed. Indeed, it could very well be that this kind of shape complementarity turns out to be a major factor in other high affinity systems as well (Wilchek & Bayer 1999).

As already mentioned, biotin dissociates remarkably slowly from avidin. In fact, the halftime for the dissociation at pH 7 is 200 days (Greene 1990). This indicates that the bound ligand must conquer a large free energy barrier in order to be released, suggesting a significant loss of binding contacts between the protein and the ligand in the transition state (Bensimon 1996, Izrailev et al. 1997). One of the major factors for this large energy barrier is thought to be the polarization of the ureido oxygen of biotin, with the oxygen existing in oxyanion resonance form. It has been suggested that the three hydrogen bonds formed by Asn-12, Ser-16 and Tyr-33 focus complementary electrostatic interactions with this ureido oxyanion and thus contribute to the energy barrier for dissociation (Weber et al. 1992). This idea is supported by the fact that chemical modification of Tyr-33 in avidin leads to reversibility of the biotin binding (Morag et al. 1996). It is also evident from the 3D-structures that once biotin has bound, conformational readjustments of avidin provide additional contributions to the binding energy. As previously mentioned the most prominent of these readjustments is the ordering of the 3-4 loop, but there are also some other minor modifications that contribute to the large energy barrier.

2.1.3 Avidin-like proteins

Since the discovery of avidin, several other sequences that bear resemblance to it have been found: streptavidins from bacteria *Streptomyces* (Chaiet et al. 1963, Bayer et al. 1995), avidin-related genes from chicken (Keinänen et al. 1988, Keinänen et al. 1994, Ahlroth et al. 2000) and the C-terminal avidin-like domain of the sea urchin fibropellins (Hursh et al. 1987, Hunt & Barker 1989, Bisgrove et al. 1991). Although the similarities of these sequences vary considerably when compared to the sequence of avidin, most of the important biotin-binding residues seem to be conserved (Table 1).

2.1.3.1 Streptavidin

Bacterial streptavidin and chicken avidin share many similar properties. Like avidin, streptavidin is a tetrameric protein that binds biotin with extremely high affinity ($K_d \sim 4 \times 10^{-14} M$) (Green 1990). Streptavidin is also a remarkably stable protein, maintaining its functional structure at high temperatures, extremes of pH, and in the presence of high concentrations of denaturants. However, there are also some notable differences between these two proteins: Avidin is a positively charged glycoprotein, whereas streptavidin is nonglycosylated and has slightly acidic isoelectric point (Green 1975). Furthermore, avidin has one disulfide bridge and two methionine residues, while streptavidin is devoid of any sulfur containing amino acids.

TABLE 1 Biotin-binding residues in avidin and the equivalent amino acids in streptavidin, avidin-related proteins and fibropellins. The most radical substitutions are underlined. Since seven *Avr*-genes and four fibropellins are currently known, some heterogeneity exists in certain amino acids.

Avidin	Streptavidin	AVRs	Fibropellins
Asn12	Asn	Asn	Asn
Ser16	Ser	Ser	<u>Asp</u>
Tyr33	Tyr	Tyr	<u>Tyr/His</u>
Thr35	Ser	Thr	Thr
Thr38	Gly	Ala	<u>Glu</u>
Ala39	<u>Asn</u>	<u>Asp</u>	<u>Arg</u>
Thr40	—	Asn	Ala/Leu/ <u>Glu</u>
Trp70	Trp	Trp	<u>Arg</u>
Phe72	<u>Arg</u>	Phe	<u>Asn</u>
Ser73	Ala	Ser	Gly
Ser75	Ser	Ser	Ser/Thr
Thr77	Thr	Ser/Thr	Thr
Phe79	Trp	Phe	Trp
Trp97	Trp	Trp	Trp
Trp110	Trp	Trp	<u>Lys</u>
Asn118	Asp	Asn	Asp

The identity between the avidin and streptavidin sequences is about 30 % with the overall similarity being 41 % (Livnah et al. 1993). Interestingly, the conserved amino acid residues are mostly confined to six homologous segments in which over 60 % of the amino acids are identical (Wilchek & Bayer 1989). Despite the relatively low sequence similarity, the three-dimensional structures of avidin (Livnah et al. 1993, Pugliese et al. 1993, Pugliese et al. 1994) and streptavidin (Hendrickson et al. 1989, Weber et al. 1989) show that the major structural elements are also conserved and the critical functional groups retained in the biotin-binding site (Table 1). Indeed, the two proteins display the same topological organization in an eight-stranded antiparallel β -barrel with a very similar fold, the only major differences being located in the exposed loop regions. The most significant of these differences is the length of the 3-4 loop, which is four residues longer in avidin than in streptavidin. As previously stated, this loop becomes ordered when biotin is bound and locks biotin into the binding pocket, and the longer loop of avidin provides tighter closure of the pocket.

In respect of the biotin-binding site, there are only two major differences between avidin and streptavidin (Livnah et al. 1993). The first is an additional aromatic group (Phe-72) in the binding pocket of avidin, which has no structural counterpart in streptavidin. The second major difference is in the hydrogen-bonding network with the valeryl carboxylate of biotin. In streptavidin the biotin carboxylate forms only two hydrogen bonds, whereas in avidin there are five. These subtle differences in the binding pocket, together with the shorter 3-4 loop, appear to form the basis of the slightly lower streptavidin affinity for the ligand (Green 1990).

2.1.3.2 Avidin-related genes and putative AVR-proteins

While trying to clone the gene encoding chicken avidin, three avidin-related genes (*Avr*) were found (Keinänen et al. 1988). Since this discovery it has been shown that there are at least seven of these *Avr*-genes and that they are located in a single cluster together with the avidin gene in the chicken male-sex chromosome Z (Keinänen et al. 1994, Ahlroth et al. 2000). The sequence identity between avidin and the *Avr*-genes in the DNA level ranges between 91 and 95 %. Interestingly, the introns are better conserved (on average 97 %) than the exons (90 %) (Wallén et al. 1995, Ahlroth et al. 2000).

Currently, it is not known whether these *Avr*-genes are expressed at the protein level, but they all contain putative promoter sequences at their 5'-ends and polyadenylation signals at their 3'-ends, suggesting the genes may be functional. In fact, it has been shown by RT-PCR that at least two *Avr*-genes, *Avr2* and *Avr3*, are transcribed in the oviduct during inflammation (Kunnas et al. 1993), but whether these mRNAs are translated into proteins is unclear. However, because the amino acids important for biotin binding are highly conserved in the predicted AVR-protein sequences (Table 1), it is likely that AVR-proteins, if expressed, would have a biotin-binding activity similar to that of avidin (Ahlroth et al. 2001).

Indeed, when recombinant AVRs were recently produced in baculovirus infected insect cells, most of them displayed a strong biotin-binding ability (Laitinen et al. 2002). Interestingly, AVR2 exhibited significantly reduced biotin binding capacity. The lowered affinity most likely results from the substitution of Lys-111 to Ile, which according to modeling results alters the shape of the biotin-binding pocket. Furthermore, all the AVRs were also shown to be extremely stable proteins like avidin. Despite these similarities the AVRs also displayed considerable differences when compared to avidin. For example, they had different pIs, glycosylation patterns and immunological properties. Consequently, these novel proteins may offer new possibilities for the applications of (strept)avidin-biotin technology.

2.1.3.3 Fibropellins

Fibropellins are epidermal growth factor (EGF) homologues found in the hyaline layer of the extracellular matrix in sea urchin embryos (Hursh et al. 1987, Bisgrove et al. 1991). These proteins harbor repeated EFG-like domains in N-terminus and a single avidin-like C-terminal domain (Hunt & Barker, 1991). Neither the function nor the three-dimensional structure of the avidin-like domain is yet known. It is also unclear whether this domain is able to bind biotin. Indeed, the preliminary results from our laboratory suggest that the avidin-like domain may lack the capability to bind biotin (Nordlund, unpublished data), despite the fact that most of the important biotin-binding residues are conserved (Table 1). However, some discrepancies can be noted with the most remarkable alteration being the replacement of Trp-110 in avidin

with positively charged lysine in fibropellins. As previously mentioned, when introduced to avidin, this change alone results in a dimeric form of avidin with reversible biotin-binding activity (Laitinen et al. 1999). Together these results suggest that the role of the avidin-like domain in fibropellins may not be to bind biotin, but to secure an appropriate structure for facilitating dimerization and thereby promote protein-protein interactions required for signal transduction.

2.2 (Strept)Avidin-biotin technology

During the last two decades the (strept)avidin-biotin system has found many uses in different fields of biology, medicine and biotechnology. Collectively these applications are known as (strept)avidin-biotin technology (Wilchek & Bayer, 1990). This technology is based on the premise that biotin can easily be attached to most biological molecules through its valeric acid side-chain without significantly changing their biological and physicochemical properties. A biologically active molecule is recognized by this biotinylated binder, which is in turn labeled with an avidin-conjugated probe (Fig. 4).

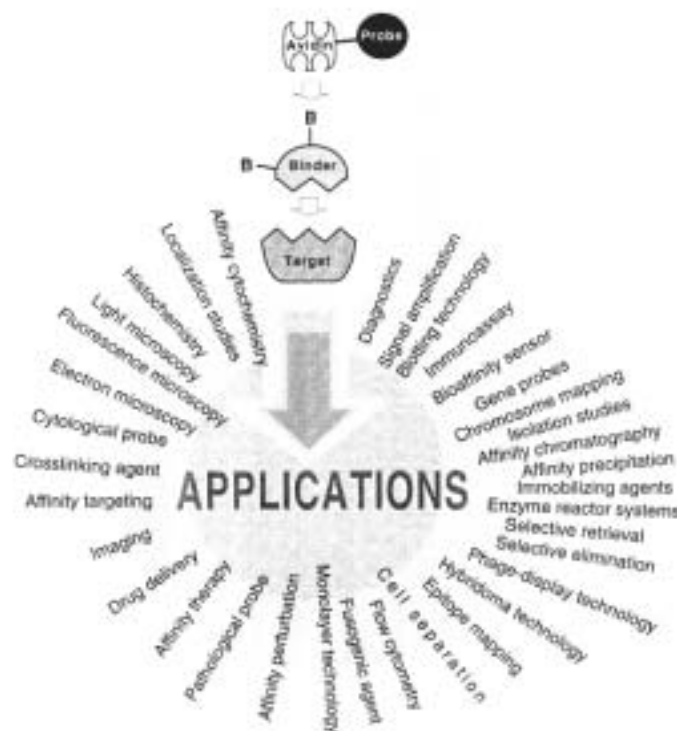


FIGURE 4 The rationale behind (strept)avidin-biotin technology. Target molecule is recognized by a biotinylated binder, which is then detected by (strept)avidin conjugated with an appropriate probe. This basic idea has been used in plethora of applications, many of which have been listed in the figure (Wilchek & Bayer 1999).

There are many reasons for the usefulness of (strept)avidin-biotin system in different applications. The main advantage is naturally the extraordinary affinity and the stability of the (strept)avidin-biotin complex, which ensures tight connection between the chosen binder and the probe. Furthermore, since one molecule of avidin (or streptavidin) can bind four biotin molecules, this causes amplification of the signal. And, as already mentioned, the labeling of different molecules with biotin is easy, and biotinylation rarely affects the biological activity of the molecule. Moreover, many biotinylating reagents, and both biotinylated and avidin-containing probes are commercially available. Yet another benefit of this system is its almost universal applicability and remarkable versatility: a given molecular target can interact with a single type of biotinylated binder that can then be analyzed in different ways using various avidin-conjugated probes. And the versatility is of course further extended through the combined use of various biotinylated binders and avidin-associated probes (Wilchek & Bayer, 1990).

The first applications of (strept)avidin-biotin technology were mostly related to isolation studies using affinity chromatography (Cuatrecasas & Wilchek 1968, Bodanszky & Bodanszky 1970). Later on avidin and streptavidin have found uses in all kinds of applications including affinity cytochemistry, localization studies, immunoassays, gene probing and diagnostics (Fig. 4) (for a review, see Bayer & Wilchek 1980, Wilchek & Bayer 1988, Wilchek & Bayer 1990 or the special (Strept)Avidin issue of *Biomolecular Engineering* vol 16, December 1999). A quite recent and exciting addition to this list is the use of the avidin (or streptavidin) in the affinity-based targeting of imaging agents and drugs (reviewed by Sakahara & Saga 1999). One example of this is the use of avidin to transfer VIPa (a cerebral vasodilator) through the blood-brain barrier (Fig. 5). In this case avidin is conjugated to a transferrin receptor antibody, which localizes the complex to the blood-brain barrier. The biotinylated VIPa binds then to avidin and the whole complex is internalized through receptor-mediated endocytosis. Furthermore, because the brain is greatly enriched in disulfide reductases, VIPa is rapidly released from the complex and is ready to affect (Bickel et al. 2001). A similar approach (avidin fused to antibody for transferrin receptor) has also been shown to target biotinylated antisense DNA for the *rev* gene of HIV-1 to the brain, which may in the future provide an effective treatment for cerebral AIDS (Penichet et al. 1999)

.Avidin and streptavidin have also been shown to be valuable tools for targeting radioactive labels *in vivo* both for imaging and treatment purposes in cancer research (Paganelli et al. 1999, Lazzeri et al. 1999, Guttinger et al. 2000). Most of these studies have utilized a pretargeting strategy called the three-step procedure. First, a biotinylated antibody is administered and is allowed to bind tumor cells expressing the antigen and to clear from the blood and normal tissues. After this avidin is administered. Some of it is bound to biotinylated antibodies whereas the unbound fraction will be cleared fast from the blood circulation. In the third step radiolabeled biotin (or an other biotinylated drug) is given and it is localized onto the tumor cells harboring avidin on their

surfaces. In this respect, an interesting paper was recently published in which avidin was fused to the macrophage scavenger receptor in order to create a fusion protein targeting system which only requires two steps: local gene transfer with the fusion protein, scavidin, and the administration of biotinylated drug (Lehtolainen et al. 2001). The exclusion of the antibody step may in some cases help to overcome the problems associated with antibodies binding to antigens also in healthy tissues or the cross-reactivity between different antigens.

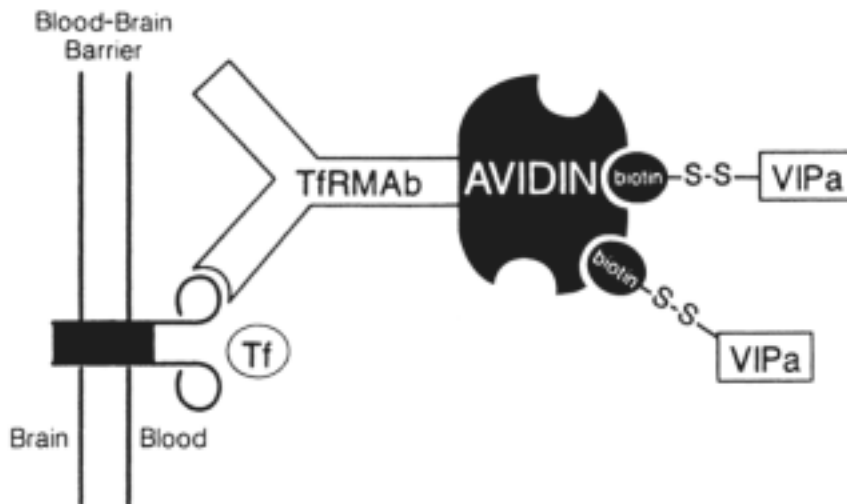


FIGURE 5 Scheme of a covalent conjugate of transferrin receptor monoclonal antibody (TfRMAb) and avidin as a brain drug transport vector. The antibody localizes the complex to the blood-brain barrier, after which avidin binds the biotinylated peptide drug, in this case VIPa. Transferrin (Tf) does not compete with the binding site of the monoclonal antibody (Bickel et al. 2001).

Unfortunately, avidin also has features that are unfavorable when considering its use in different applications. The strong positive charge of avidin can lead to non-specific binding to negatively charged molecules such as nucleic acids. The presence of a carbohydrate moiety can also cause unwanted interactions with lectin-like molecules (Bayer & Wilchek 1994). Furthermore, the high pI and the sugar residues together are also responsible for the rapid clearance of avidin from the blood stream, which can be a detriment for its use in drug targeting (Kang & Pardridge 1994, Kang et al. 1995, Yao et al. 1999). For these reasons streptavidin has been the protein of choice in many applications, since it is nearly neutral and devoid of sugars. But streptavidin has also its disadvantages. For example, it contains an Arg-Tyr-Asp sequence (Alon et al. 1990), which is highly reminiscent of the universal cell surface recognition sequence (Arg-Gly-Asp) present in various adhesion molecules (fibronectins, fibrinogens and collagens). Because of this sequence, streptavidin interacts in a biotin-independent manner with integrins and related cell surface receptors (Alon et al. 1992). Moreover, streptavidin is highly immunogenic, which considerably

limits its use in drug targeting (Chinol et al. 1998). Streptavidin is also more expensive than avidin and contains less lysine residues for potential attachment of the probes.

To overcome the problems associated with the non-specific binding characteristics of avidin, the use of high salt concentrations or high pH was originally suggested (Bussolati & Gugliotta 1983). The use of other basic proteins (e.g. lysozyme) for blocking the negatively charged groups was also studied (Bayer et al. 1987). In many cases, these methods were employed with success, but neither was found to be universally applicable. Later on chemical modifications to reduce the charge of avidin have brought some relief to this problem, and two this kind of avidin derivatives are commercially available: NeutrAvidin (Pierce, Rockford, IL, USA) and NeutraLite Avidin (Belovo Chemicals, Bastogne, Belgium) which is also enzymatically deglycosylated. Both of these variants have been shown to exhibit clearly reduced non-specific binding to negatively charged materials (Bayer & Wilchek 1994). Chemical modifications of this kind have also been used to improve pharmacokinetics and biodistribution of avidin (Rosebrough & Hartley 1996, Chinol et al. 1998).

2.3 Protein engineering

During the past few years, there has been a growing interest towards the problem of how to create proteins equipped with coveted activities capable of performing in user-defined conditions. Traditionally, the answer to this problem has been the use of pre-evolved diversity in nature by screening of proteins from various microbes that have become adapted to extreme environmental surroundings, such as high a temperature or salt concentration (Robertson et al. 1996, Madigan & Marrs 1997). However, the mainstream attention has now shifted to two fundamentally different ways of creating new activities by direct engineering of protein function. The first one involves *de novo* design of a polypeptide chain that folds correctly and has the desired activity, and the second one comprises redesigning the properties of pre-existing proteins.

Although, there have been some recent advances in the *de novo* design of certain protein motifs, like α -helical bundles (Betz et al. 1997, Schafmaster et al. 1997), triple-stranded β -sheets (Kortemme et al. 1998, Griffiths-Jones & Searle 2000), coiled-coil structures (Severin et al. 1997) or helical hairpins (Ramagopal et al. 2001), the knowledge to consistently design enzymatic activity is still lacking. The two major obstacles still remaining are problems concerning the theoretical calculations on the reaction dynamics and, particularly the inadequate understanding of protein folding. Nevertheless, the current designed proteins provide excellent model systems for the elucidation of the fundamental principles that will eventually able us to create novel enzymes, in particular for reactions not catalyzed by nature. However, since the *de novo*

design of enzymes is still an unrealistic target, much effort has been put into protein engineering for creating novel proteins and also elucidating the structure-activity relationship. The rationale behind the idea of creating new proteins by modifying existing ones lies in the fact that the number of individual proteins thought to occur in nature (10^5) is much bigger than the number of individual protein folds, which is estimated to be around 10^3 (Zhang & DeLisi 1998). The difference in these numbers is due to the fact that many proteins share common folds, which in turn means that proteins with a similar fold can perform very different tasks. Therefore it should be possible (at least in theory) to engineer new activities for pre-existing structural frames by changing appropriate amino acids.

Modifying properties of a certain protein can be approached from different directions. Rational design defines one end of the spectrum of approaches used to engineer any given protein. In this approach, information concerning the structure and the function of the protein is used as a basis for altering its properties in a predictable fashion. At the other end of this engineering spectrum is the random, or directed evolution approach. This approach requires little information about the protein to be engineered. Instead, the success of this random method relies on the ability to screen large libraries of variant proteins to identify the desired activity. Regardless of the method chosen, the gene encoding the protein of interest, a suitable expression system and a sensitive detection system are prerequisites. Furthermore, careful analysis of the resulting protein is necessary for additional knowledge about the molecule, refining the experimental design and providing information about the general engineering principles (Bornscheuer & Pohl 2001).

2.3.1 Rational design

As already mentioned, rational design usually requires both the availability of the structure and the knowledge about the relationships between sequence, structure and mechanism/function, and is therefore very information-intensive. On the other hand, more and more 3D-structures of proteins are available and the number of sequences stored in the data banks is increasing enormously, making rational design easier. An elegant example of how this approach can be used to alter the substrate specificity of an enzyme was presented by Shokat and coworkers (Shah et al. 1997). In order to identify the direct substrates of v-Src kinase, they engineered the nucleotide-binding site of this enzyme so that it was able to bind and efficiently catalyze the phosphotransfer of an ATP analog not utilized by any other kinase while still maintaining its original substrate recognition properties. This was done by changing two key amino acids (Val-323 and Ile-338, identified from the structures of two homologous kinases) in the nucleotide-binding site. Substitution of these two bulky residues with alanines created space in the binding site, which allowed the kinase to bind and utilize ATP analog derivatized at the N⁶-position (Fig. 6). By using a ³²P-labelled ATP analog they were then able to identify the targets of the v-Src kinase, since no other kinase was able to utilize this molecule. Besides changing substrate

specificity, the rational design approach has also successfully been used, for example, in the stabilization of proteins such as thermolysin-like protease (Van den Burg et al. 1998) or hen lysozyme (Ueda et al. 2000) towards thermoinactivation and for re-engineering the catalytic mechanisms of enzymes like aspartate aminotransferase (Graber et al. 1999) or T4 lysozyme (Kuroki et al. 1999).

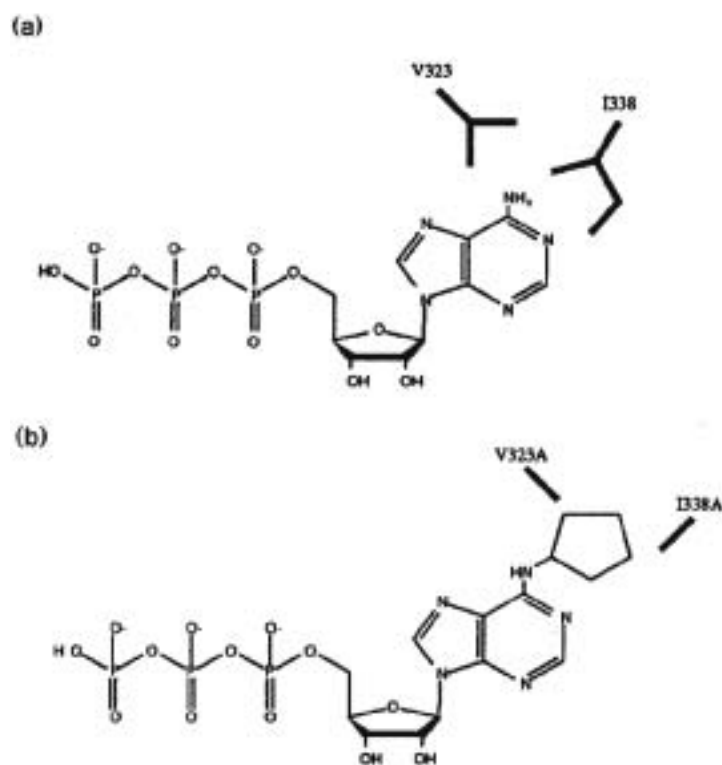


FIGURE 6 (a) The structure of ATP with the v-Src kinase amino acids targeted for mutagenesis (Val-323 and Ile-338). (b) The N⁶-(cyclopentyl) ATP analog shown in context of the engineered v-Src (V323A and I338A) (Harris & Craik 1998).

Although the structure-based rational protein design has yielded some success, this approach has also its drawbacks. In many cases the effects of multiple mutations are difficult to predict, even with the structural data in hand. For example, changes in the residues of the active site of an enzyme often produce the desired results in terms of specificity, but at a large cost to the catalytic rate (Shanklin 2000). Furthermore, key amino acids that are important for the activity of the protein are not necessarily located near the active site and therefore are not easily identified from the structure. Structural prediction and site-directed mutagenesis of target residues can also be labor-intensive and time-consuming. Despite these problems, however, the rational approach is often a viable alternative, when the detailed knowledge of the protein structure and function are available.

2.3.2 Directed evolution

In nature many proteins with distinct functions have evolved one from another. By using directed evolution, researchers attempt to mimic these natural processes by which protein variants emerge and are selected within living systems. Basically this approach involves two steps (Fig. 7). First the target gene is subjected to random mutagenesis either by error-prone PCR (Fromant et al. 1995) or by fragmentation followed by reassembly (Stemmer 1994). In the second step, the improved genes are genetically recombined (shuffled) to create a new gene library that contains combinations of the mutations isolated in the first step, after which improved genes are selected again. This cycle can then be repeated several times to reach the final outcome. This approach has turned out to be useful method for the evolution of single gene products with, for example, altered substrate specificity (β -fucosidase from β -galactosidase) (Zhang et al. 1997), improved protein folding (GFP) (Cramer et al. 1996) and pathways with improved function (arsenate detoxification pathway) (Cramer et al. 1997).

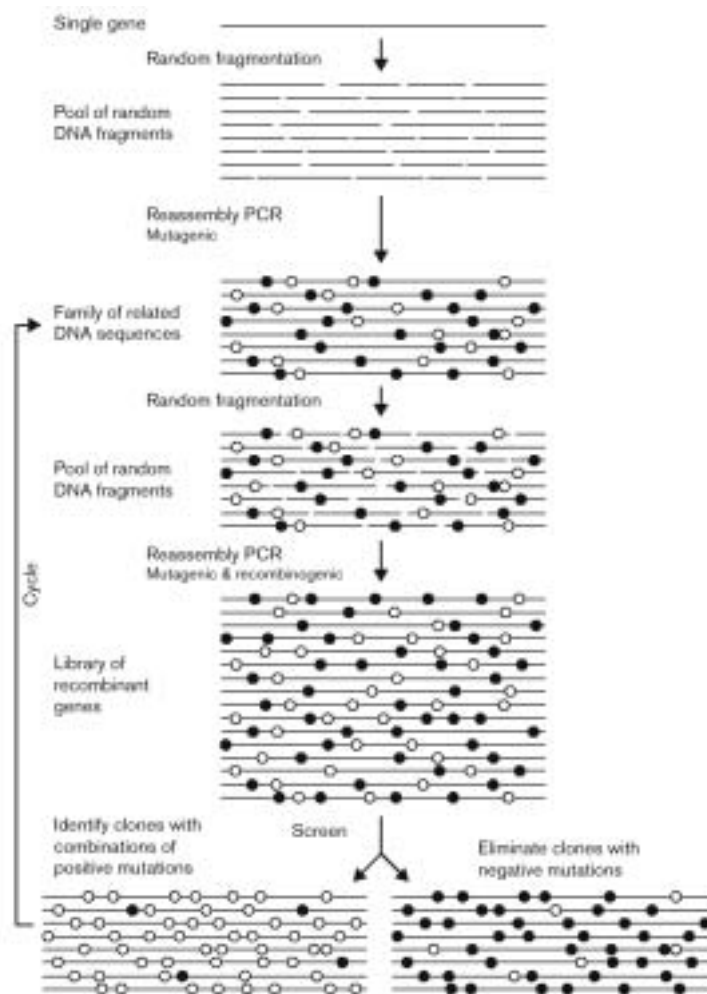


FIGURE 7 Summary of the principles of gene shuffling. Mutations result in positive (○) or negative (●) phenotypes (Shanklin 2000).

To succeed in the use of directed evolution, it is important to select the target gene that encodes protein with properties as close as possible to the coveted feature in order to minimize the evolutionary distance (Arnold 1998). Another prerequisite for successful engineering is a screening protocol that permits a large number of candidate clones to be appraised for the traits of interest (Zhao & Arnold 1997). Where applicable, biological selection (either complementation of auxotrophy or resistance to cytotoxic agents) offers a potent way of screening libraries and isolating improved proteins (MacBeath et al. 1998, Oue et al. 1999). However, the use of phenotypic selection is limited only to events that have direct biological relevance. Solid-phase screening offers another efficient way to evaluate libraries expressed in microorganisms (Matsumura et al. 1999, Joo et al. 1999). This method relies on product solubilization following the enzymatic reaction, which in turns leads to a zone of clearance, a fluorescent color or strongly absorbing product. Unfortunately, many assays cannot be executed in a solid-phase format, meaning that the clones must be grown and assayed in microtiter wells, which is more time-consuming. The third common approach to screen libraries is to use phage display. Here the protein variants are expressed on the surface of the filamentous bacteriophage and then selected on the basis of the ligand binding (reviewed in Forrer et al. 1999). Protein libraries have also been displayed on cell surfaces and analyzed with fluorescence-activated cell sorting (Daugherty et al. 2000, Holler et al 2000). Recently also *in vitro* display systems such as ribosome display (Hanes & Plückthun 1997, He & Taussig 1997) and mRNA display (Roberts & Szostak 1997, Nemoto et al. 1997) have been developed. These systems make use of a physical link between the messenger RNA and the nascent polypeptide chain during the translation to couple genotype and phenotype. The biggest advantage of these *in vitro* techniques is that very large libraries up to 10^{14} can be constructed since the number of molecules that can be handled is not limited by cellular transformation efficiencies.

Although single gene shuffling has successfully been used for evolving many different properties of various different proteins, the shuffling of multiple homologous DNA sequences has proven to be even more powerful (Minshull & Stemmer 1999). This was first demonstrated by Cramer and co-workers (Cramer et al. 1998) in a study where they shuffled together four cephalosporinase genes. The result was a 270-540 fold improvement in enzyme activity in a single round of shuffling, whereas single gene shuffling of the four genes independently only yielded eight-fold improvements. The clone exhibiting the highest activity had 33 point mutations and resulted altogether from seven crossovers of the starting genes. The large evolutionary distance between the best clone and any one of the individual starting genes implies that this particular protein would have never been found, if structure-based rational design or random mutagenesis approach had been used. Other successful examples of multiple gene shuffling include, for example, directed evolution of herpes simplex virus thymidine kinases (Christians et al. 1999), and shuffling of subgenomic sequences of subtilisin (Ness et al. 1999). The subtilisin study is

particularly interesting because the authors describe the evolution of multiple properties of the enzyme simultaneously.

Finally, it has been recently shown that rational (structure-based method) and directed evolution approaches can be used as complementary tools in protein design (Altamirano et al. 2000). In this study these two techniques were used together to engineer a new catalytic activity, phosphoribosylanthranilate isomerase (PRAI), from the α/β -barrel scaffold of indole-3-glycerol-phosphate synthase (IGPS). The basic idea was first to use rational design to create an “intermediate” structure that the authors assumed would be near the final design. Directed evolution was then applied to generate subtle changes that fine-tuned the protein packing and functions. Indeed, the best clone was shown to have even higher PRAI-activity than the wild-type enzyme from *E.coli*. Besides proving that these two philosophically different approaches can be utilized synergistically, this study is also important because it shows that the α/β -scaffold (present in approximately 10% of all proteins) can be redesigned to harbour new specifications.

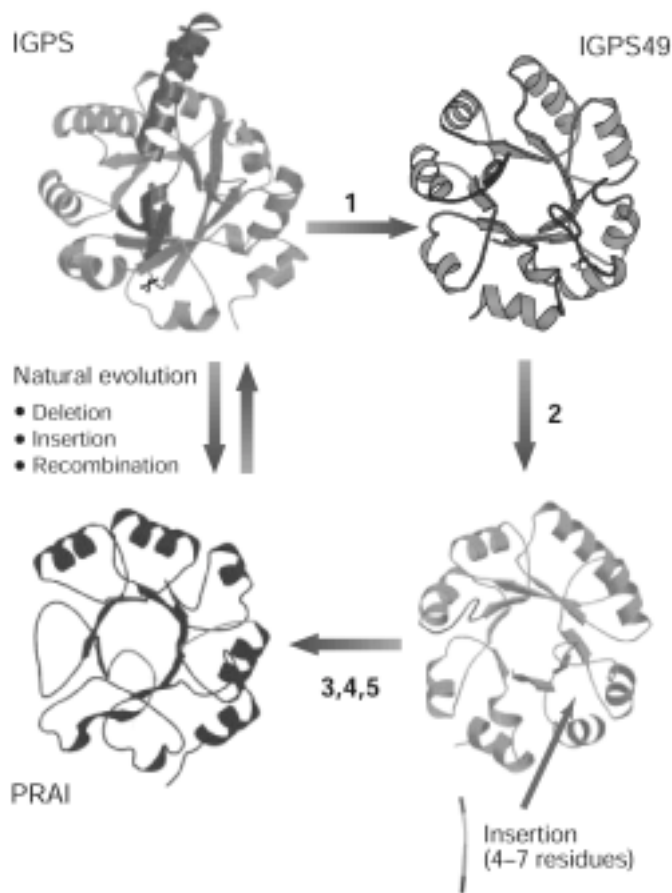


FIGURE 8 Ribbon representation of the experimental strategy for evolving a new function in the IGPS scaffold. Top left: initial IGPS scaffold. 1 deletion of 48 amino acids from the N-terminus, yielding IGPS49. 2 deletion of 15 amino acids from loop $\beta 1\alpha 1$ and replacement with 4-7 residues from PRAI. 3 introduction of Asp residue in position 184 and replacement of loop $\beta 6\alpha 6$ by PRAI consensus sequence. 4 *in vivo* selection by complementation of PRAI-deficient strain. 5 *in vitro* recombination (DNA shuffling and StEP PCR) to fit barrel shape and improve its function (Altamirano et al. 1999).

3 AIM OF THE STUDY

Chicken avidin is a fascinating protein in many respects. The extremely tight biotin-binding affinity of avidin has been utilized in a plethora of applications including purification, labeling, diagnostic and drug targeting systems. Furthermore, this strong interaction with biotin has also generated considerable protein chemical interest, particularly as an attractive model system for investigating protein-ligand interplay. Avidin also displays one of the greatest thermal stabilities observed for a protein from a mesophilic organism: in the presence of biotin, it maintains its functional structure completely at the ordinary water boiling temperature.

The aims of this study in general were two-fold. Firstly, from the protein chemical point of view we were interested to know what kind of changes in the amino acid sequence avidin can tolerate when its different properties (charge, oligomerization, biotin-binding) are modified, while still retaining its functionality. Our second perspective was more practical, i.e. by modifying the characteristics of avidin we wanted to create new tools for (strept)avidin-biotin technology in order to further expand this already versatile system. In more detail the specific aims of this study were:

1. To create a series of avidin charge mutants with lowered pI and to study whether these mutations effect the biotin binding or the stability of the avidin tetramer.
2. To improve the non-specific binding characteristics of avidin in different applications by creating a non-glycosylated and acidic variant of avidin with high affinity for biotin.

3. By means of rational design to disrupt the avidin tetramer into monomeric components, which still are capable of biotin binding and this way gain more insight into the formation and the stability of the avidin tetramer.
4. To investigate the role of Tyr-33 of avidin in biotin binding and by mutating this residue to create tetrameric avidin variants with more reversible biotin binding characteristics.

4 SUMMARY OF MATERIALS AND METHODS

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

4.1 Site-directed mutagenesis and construction of recombinant baculoviruses

Mutagenesis of avidin cDNA (Gope et al. 1987) was performed by the PCR-based megaprimer method (I-IV) (Sarkar & Sommer 1990). After the second PCR amplification the fragments were digested with *Bgl*II and *Hind*III and then subcloned into a *Bam*HI/*Hind*III digested pFastBAC1 donor vector (Gibco BRL). The constructs were then transformed into JM109 *E. coli* cells and the mutations confirmed by dideoxynucleotide sequencing. The recombinant baculoviruses were produced using the Bac-to-Bac baculovirus expression system (I-IV) (Gibco BRL): First, the recombinant baculovirus genomes were generated by site-specific transposition in DH10Bac *E. coli* cells. After that the virus genomes were purified and transfected into Sf9 cells in order to create functional baculoviruses. The primary virus stocks were amplified for large-scale production of mutant proteins and the titers of the stocks were determined by a plaque assay procedure.

4.2 Production and purification of avidin mutants

Spodoptera frugiperda, Sf9, insect cells (ATCC CRL-1711) were grown in suspension cultures in serum-free culture medium (Sf 900IISFM, Gibco BRL)

and infected with different recombinant baculoviruses at m.o.i of 0.5-2 pfu/cell (I-IV). The culture medium was changed from biotin-containing to biotin-free either at the start of the infection or 24 hours post infection. After four days of infection the insect cells were broken by lysis buffer and sonication. The mutant proteins were then purified from the soluble fraction by pH-dependent affinity chromatography using 2-iminobiotin agarose (Sigma B-4507).

4.3 Protein analysis

Electrophoretic analysis was carried out with 15% (w/v) SDS-PAGE using the standard procedure described by Laemmli (1970). After electrophoresis, proteins were either stained with Coomassie brilliant blue or blotted onto nitrocellulose membrane for immunostaining according to Airene et al. (1997). Isoelectric focusing was performed using polyacrylamide gels with a pH gradient ranging from three to ten and following the run the proteins were visualized by Coomassie staining (I). The quaternary status of avidin and the different mutants was analyzed either by FPLC on a Superose 12 column (Pharmacia) using an LK HPLC system (I-III) or by a Shimadzu HPLC system with a SuperdexTM 200 HR 10/20 column (IV). Chromatography was carried out using the same buffer and ionic strength in the equilibration and running phases at the flow rate of 0.5 ml/min. Bovine γ -globulin, BSA, native avidin, ovalbumin, carbonic anhydrase, ribonuclease and cytochrome c were used as molecular weight markers to calibrate the column.

4.3.1 Stability assays

In order to study the thermal stability of the avidin mutants (I-IV), the purified proteins in the presence or absence of an excess of biotin were incubated at selected temperatures for 20 minutes, before being subjected to SDS-PAGE as described by Bayer et al. (1996). The gels were stained using Coomassie brilliant blue and the stability of the proteins was followed by dissociation of the tetramer to the monomeric form. The sensitivity to proteolytic digestion was tested by mixing avidin or the mutant proteins in the presence or absence of biotin with proteinase K at a w/w ratio of 1:50 (II-IV). The reaction mixture was incubated at 37°C and samples were taken at the designated time intervals. The samples were analyzed by SDS-PAGE and the amount of intact protein in each case in the expected band was determined by densitometry and compared to that of an untreated control sample.

4.3.2 Biotin-binding activity

Biotin-binding activity was preliminarily demonstrated with avidin mutants by using affinity purification with 2-iminobiotin-agarose. To further characterize

the biotin-binding ability of different mutants, an optical biosensor instrument (IASys Manual+, Affinity Sensors) was used (I-IV). The measurements were carried out using either a commercial biotin cuvette (Affinity Sensors) or by immobilizing 2-iminobiotin onto the carboxymethyl dextran cuvette using N-hydroxysuccinimide activation. The binding of various concentrations of avidin or avidin mutants onto 2-iminobiotin surface was measured in a 50 mM borate buffer (pH 9.5) containing 1 M NaCl₂ at room temperature. The measurements with the biotin cuvette were carried out using PBS with 1M NaCl as a binding buffer at room temperature. The biosensor data was analyzed and the kinetic parameters of the different mutants were calculated using the Fast Fit program package (Affinity Sensors). To test the reversibility of biotin binding, two assays were used: a competitive biotin binding enzyme-linked immunosorbent assay (III) and a competitive biosensor assay using biotin cuvette (III and IV).

4.3.3 Non-specific binding assay

The non-specific binding characteristics of avidin and different mutants were examined by a slot-plot assay (I, II). Successive dilutions of salmon-sperm DNA or pGEM plasmid DNA were fixed to the slots and the dried strips were quenched using 5 × Denhart's solution. The desired avidin or mutant sample was then added and incubated at room temperature for 90 min. The strips were then washed and stained immunochemically as previously described.

The test for non-specific binding to different cells (human platelets and lymphocytes, mouse hepatocytes, and *E. coli* strain HB101) was performed as follows (II). The cell suspension was allowed to dry in the slots by applying a gentle vacuum, thus fixing the cells to the nitrocellulose membranes. The slot blots were quenched using 0.5% BSA, challenged by the chosen avidin/mutant sample. The nitrocellulose membrane was removed from the apparatus, quenched again and the bound protein was detected immunochemically.

4.3.4 Preparation of Avm-Y33H column

A protein sample (0.5 mg) was combined with 700 mg of CNBr-activated Sepharose resin. After two hours the resin was washed with water and PBS. The reversibility of the column was tested with biotinylated BSA. Two ml of B-BSA solution (0.25 mg/ml) was applied to the column after which it was rinsed with 50 mM sodium citrate (pH 4). A biotin solution (0.6 mM biotin in 50mM Tris buffer, pH 8.4) was then applied and the amount of the eluted protein was determined.

4.4 Computer programs utilized

The GCG program package (Genetic Computer Group, Madison, WI, USA) was used to calculate the theoretical molecular weights for the mutant proteins (I-IV) and to determine the theoretical isoelectric points of the charge mutants (I). In study III the figures of native avidin were produced with the MOLSCRIPT program using avidin coordinates from the PDB database.

5 REVIEW OF THE RESULTS

5.1 Reduced charge avidin mutants (I)

A series of six avidin charge mutants was constructed in order to reduce the positive charge of native avidin. Site-directed mutagenesis was used to change some of the basic amino acids of avidin to either neutral or acidic ones. The theoretical pI values for these mutants varied from 9.9 to 4.7 as determined from the amino acid sequences. The selection of amino acids for lowering the isoelectric point (Table 2) was based on sequence comparison of avidin, streptavidin and the putative avidin-related proteins combined with knowledge of the three-dimensional structure of avidin.

TABLE 2 Avidin charge mutants. The mutant proteins were named according to the experimental pI. The actual pI for native avidin was not determined since its pI is over 10, which is over the limits of the pH gradient used in this study.

Name	Mutations	pI calculated	pI experimental
Avd	none	10.4	n.d.
AvdpI9.4	R122A, R124A	9.9	9.4
AvdpI9.0	R26N, R59A	9.3	9.0
AvdpI7.9	R2A, K3E, K9E	8.1	7.9
AvdpI7.2	K3E, K9D, R122A, R124A	7.2	7.2
AvdpI5.9	R2A, K3E, K9E, R122A, R124A	5.9	5.9
AvdpI4.7	R2A, K3E, K9E, R26N, R59A R122A, R124A	4.7	4.7

In a previous study (Airenne et al. 1997) we have shown that the baculovirus expression system is an efficient strategy with which to produce soluble and

functional recombinant avidin with reasonably good yields. All the charge mutants were also produced in soluble form with yields that were similar to those of wild-type avidin. The resultant mutant proteins were purified in one step with affinity chromatography using 2-iminobiotin agarose. Biotin-free culture medium was used for the expressions, since if biotin is present it will block the binding sites in avidin/mutants, preventing the subsequent affinity purification.

Isoelectric focusing with a pH gradient from 3 to 10 was used to resolve the pIs of the different charge mutants (I, Fig. 1). The experimental values were in good agreement with pIs deduced theoretically from the amino acid sequences (Table 1). In order to study the quaternary structure of the charge mutants, FPLC was carried out. When the elution profiles were compared with those of molecular weight standards and native avidin, it was clear that all these variants formed stable tetramers with molecular weights similar to that of native avidin.

The biotin-binding ability of the mutant proteins was analyzed with an IAsys optical biosensor instrument. All the mutants bound to an immobilized biotin surface in an irreversible manner and no dissociation was observed. This result already indicated that the biotin binding of all the mutants was extremely strong - resembling the tenacious binding of wild-type avidin. In order to further investigate the binding characteristics of different mutants, we decided to use a 2-iminobiotin surface, since avidin binds this biotin analog with lower, readily measurable affinity. The dissociation constant for wild-type avidin was calculated to be 2.0×10^{-8} M and the K_d values for the different mutant proteins varied from 4.5×10^{-7} M to 3.5×10^{-8} M (I, Table 1). These results together with the irreversible binding to the biotin surface suggest that all the charge mutants exhibit biotin-binding ability similar to that of wild-type avidin.

To test the thermal stability of the charge mutants a SDS-PAGE assay was carried out. The proteins, with or without biotin, were subjected to different temperatures between 25 °C and 100 °C in SDS-containing buffer. Without biotin native avidin begins to dissociate into monomers at temperatures over 57 °C (I, Fig. 3A) whereas biotin-saturated form requires temperatures near 100 °C to unfold (I, Fig. 3B). Of the six charge mutants, AvdpI4.7 (I, Fig. 3C and Fig. 3D), AvdpI9.0 and AvdpI9.3 displayed dissociation profiles similar to that of native avidin. In contrast, AvdpI5.9, AvdpI7.2 and AvdpI7.9 (Fig. 9) dissociated into monomers already at room temperature in the absence of biotin. However, the adding of biotin restored their stability; i.e., when biotin was present these proteins also required temperatures near 100 °C for dissociation.

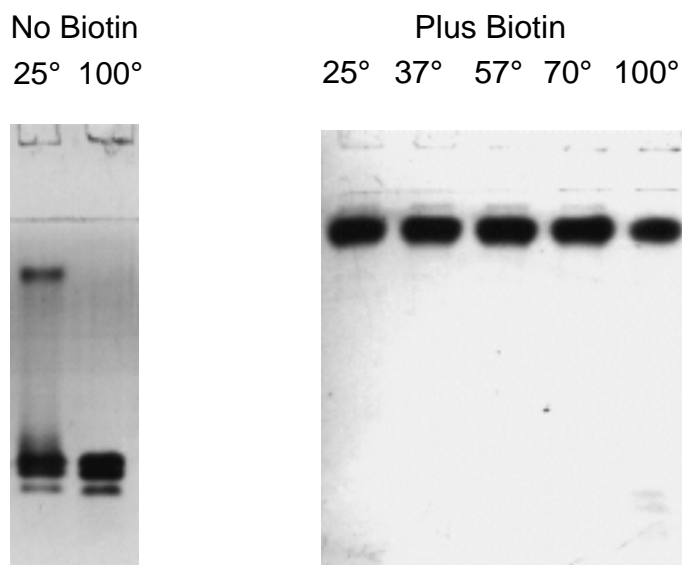


FIGURE 9 Thermal stability of AvdpI7.9 in the absence and in the presence of biotin (Coomassie brilliant blue-stained SDS-PAGE gel). The protein samples were incubated at the designated temperatures for 20 minutes in SDS-PAGE sample buffer before being subjected to electrophoresis.

5.2 A non-glycosylated and acidic mutant of chicken avidin (II)

In order to produce a recombinant non-glycosylated avidin mutant (ngAvm), the Asp-17 of avidin was mutated to Ile. Isoleucine was selected since sequence comparison showed that all avidin-related proteins, except AVR7, bear Ile in the corresponding position (II, Fig 1). To further modify the characteristics of avidin, this non-glycosylated mutant, ngAvm, was combined with the previously engineered charge mutant Avm-pI4.7 (I), and the ensuing protein was named ngAvm-pI4.7. Of the six different avidin charge mutants constructed, Avm-pI4.7 was chosen because it was shown to be an extremely stable protein and its biotin-binding ability was similar to that of the wild-type avidin.

The mutant proteins were produced in insect cells using baculovirus expression and purified with 2-iminobiotin agarose. The final yields of these mutants were between 7 and 9 mg per liter of culture, indicating that the mutations had little effect on the expression when compared to that of wild-type avidin. When the proteins were analyzed with SDS-PAGE, ngAvm behaved similarly to native avidin, forming aggregates at lower temperatures that failed to penetrate the separation gel (II, Fig. 2). However, when the temperature is raised, the aggregates dissociate and the proteins migrate as monomers. Interestingly, under similar non-denaturing conditions, both acidic mutants Avm-pI4.7 and ngAvm-pI4.7 migrated in the separating gel as a tetramer (II, Fig. 2).

The biotin-binding characteristics of ngAvm, Avm-pI4.7 and ngAvm-pI4.7 were analyzed with an IAsys optical biosensor. However, the binding of all the mutant proteins to immobilized biotin was so tight that practically no dissociation was seen. Therefore a biotin analog, 2-iminobiotin, was again used, since it exhibits a substantially lower affinity for avidin. When the binding curves of the different mutants were analyzed, it was shown the kinetic parameters (k_{on} , k_{off} , K_d) of binding to 2-imino biotin were similar to those of wild-type avidin (II, Table 2) suggesting that their biotin-binding activity was relatively unaffected by the changes in the amino acid sequence.

The thermal stability of ngAvm, Avm-pI4.7 and ngAvm-pI4.7 was tested by means of the SDS-PAGE assay, as previously described. Both ngAvm and Avm-pI4.7 displayed extreme stability comparable to that of wild-type avidin. In other words the tetramer-to-monomer transition in the absence of biotin occurred in these proteins over the temperature range of 50° and 70° C, whereas with biotin temperatures near 100° C were required for the dissociation to begin (II, Fig. 3). In the case of ngAvm-pI4.7, a slight decrease in the stability, both with and without biotin, was observed, but it still exhibited high degree of stability.

The stability of these proteins was also studied by testing their sensitivity to the proteolytic enzyme proteinase K in the absence and in the presence of biotin. Without biotin wild-type avidin is susceptible to slow proteolysis by proteinase K, whereas with biotin it is completely resistant (II, Fig. 4). The glycosylated mutant Avm-pI4.7 behaved similarly to wild-type avidin, but both the non-glycosylated proteins, ngAvm and ngAvm-pI4.7, were degraded at clearly faster rate in the absence of biotin (II, Fig. 4). Nevertheless, the binding of biotin stabilized these proteins as well and rendered them resistant to proteinase K.

The non-specific binding of avidin and the mutant proteins to DNA and to four different cell types was studied with a slot-plot assay. Owing to its high positive charge, avidin bound strongly to negatively charged DNA, as did ngAvm, since it has the same pI as wild-type avidin. On the other hand, both the acidic mutants, Avm-pI4.7 and ngAvm-pI4.7, showed no binding to DNA (II, Fig. 5A). Wild-type avidin and ngAvm also displayed strong non-specific binding to the four different cell types used in this study. However, reducing the positive charge of avidin also drastically lowered the non-specific binding to these cells, since Avm-pI4.7 showed only weak binding to two cell types (i.e. hepatocytes and lymphocytes). Removing the carbohydrate moiety from the Avm-pI4.7 (i.e. ngAvm-pI4.7) further reduced its binding to the different cell types to such an extent, that no binding could be detected.

5.3 Two monomeric avidin mutants that undergo biotin-induced tetramerization (III).

The aim of this study was to dismantle the avidin tetramer into smaller components. We have previously shown that changing Trp-110 (located between the interfaces one and two) to Lys results in a dimeric form of avidin with reversible biotin binding (Laitinen et al. 1999). In the present work, we concentrated on the interfaces between subunits one and three and one and four. The target residues for mutagenesis were chosen on the bases of the three-dimensional structure of avidin, taking into consideration their importance for the particular interface. First, the three hydrophobic amino acids forming the interface between subunits one and three were changed to alanine. The resultant mutant was named Avm-3 (III, Table 1). Secondly, Asn-54 was mutated to Ala (Avm-4a), in order to disrupt the net of hydrogen bonds that this residue forms between the interfaces one and four. Moreover, these two interface mutants were combined in Avm-[3,4a]. In addition, we constructed another mutant Avm-[3,4b], which had all the above-mentioned changes and an additional Asn69Ala mutation. All the mutant proteins were expressed in baculovirus-infected insect cells and purified in high yields with affinity chromatography using 2-iminobiotin as a capturing ligand.

In order to study the quaternary status of the different mutants, a gel filtration FPLC was run and the molecular weights of the different mutants were compared to those of avidin and molecular weight standards (III, Fig. 6). Of the four different mutants, Avm-3 and Avm-4a behaved similarly to native avidin, forming stable tetramers both with and without biotin. However, in the absence of biotin, the observed masses for Avm-[3,4a] and Avm-[3,4b] were 13,990 Da and 14,280 Da, respectively, indicating that the proteins exist in monomeric form. Interestingly, in the presence of biotin the molecular weight of both proteins was much higher (58,900 Da for Avm-[3,4a] and 55,960 Da for Avm-[3,4b]), corresponding to that of the tetrameric form of native avidin.

A reversibility assay based on optical biosensor technology was used to evaluate the biotin-binding ability of the mutant proteins (III, Fig. 2). Of the four mutants only Avm-4a exhibited similar behavior to that of native avidin showing complete irreversibility. The three other mutants displayed reversibility between 30 and 45 % with Avm-[3,4a] exhibiting the loosest binding. To further test the binding characteristics of the mutants, their binding to a 2-iminobiotin surface was studied using the IAsys biosensor. The measurements indicated that the affinity of the mutants Avm-3 and Avm-4a was similar to that of native avidin (K_d 2×10^{-8} M), the actual dissociation constants being 7.4×10^{-8} M and 2.7×10^{-8} M, respectively (III, Table 2). In the case of the two other mutants, Avm-[3,4a] and Avm-[3,4b], accurate K_d values could not be determined with the biosensor due to monomer-tetramer transition accompanying the biotin binding. Nonetheless, in qualitative terms these proteins also displayed strong binding to 2-iminobiotin.

When the thermal stability of the different mutant proteins was tested with the previously described SDS-PAGE assay, it was shown that all the four mutants exhibited lowered stability in the absence of biotin as compared to that of native avidin (III, Fig. 4). Avm-3 and Avm-4a displayed a partial tetrameric structure in SDS-PAGE at ambient temperatures, whereas Avm-[3,4a] and Avm[3,4b] migrated completely as monomers, even at room temperature. However, when the proteins were saturated with biotin, all four mutants showed stability characteristics comparable to those of wild-type avidin. In other words, when biotin was present, all the mutants migrated as stable tetramers up to temperatures near 100 ° C. These results are in a good agreement with the FPLC data presented above.

Similar results were also obtained when the stability of the mutant proteins was tested with proteinase K treatment. As in the case of thermal stability, all the mutants exhibited reduced stability in the absence of biotin, i.e. they were degraded at a substantially faster rate than native avidin (III, Fig. 5). However, when biotin was added, complete resistance was restored to three of the mutants (Avm-3a, Avm-4a and Avm-[3,4b]) and the stability of Avm-[3,4a] was also reinforced so that 75 % of the protein remained intact after 16 hours of incubation with the enzyme.

5.4 Avidin mutants with reduced biotin-binding capacity (IV)

In order to produce avidin mutants with lowered (i.e. more reversible) biotin-binding ability, we targeted Tyr-33 of avidin for mutagenesis. Tyr-33 was selected, since this amino acid forms one of the hydrogen bonds in the triad with the carbonyl group of the ureido ring of biotin (Livnah et al. 1993). Furthermore, it has been shown that nitration of this residue results in an avidin variant (called nitro-avidin) with reversible biotin binding (Morag et al. 1996). Therefore we replaced this tyrosine with four different amino acids (Avm-Y33F, Avm-Y33A, Avm-Y33Q and Avm-Y33H) to study what kind of effects these substitutions would have on biotin binding or on the stability of the avidin tetramer.

All four mutants were produced successfully in baculovirus-infected insect cells. Avm-Y33F, Avm-Y33A and Avm-Y33H were efficiently purified with affinity chromatography using 2-iminobiotin agarose, whereas Avm-Y33Q displayed clearly reduced binding to this ligand so that the purification efficiency of this protein was less than 50 %.

When the reversibility of the biotin binding of avidin and different mutants was studied, it was shown that at neutral pH the binding was practically irreversible in all cases. However, if the pH was raised to nine, Avm-Y33H exhibited substantial reversibility, meaning that about half of the bound protein was released when free biotin was added (IV, Fig. 2). Avm-Y33Q was also shown to display some reversibility (29%), whereas Avm-Y33F and Avm-

Y33A continued to bind to biotin surface in almost irreversible fashion even under these conditions. We have also tested the binding of biotinylated BSA to Avm-Y33H-Sepharose column. The preliminary results suggest that above pH 8 the binding is indeed partly reversible and that about 50 % of the bound biotinylated BSA could be released with free biotin (unpublished results)

We also tested the binding of these avidin variants to a 2-iminobiotin surface to obtain more information about possible differences in binding characteristics. In the case of Avm-Y33Q some specific binding was observed, but we were unable to measure the actual kinetic parameters because the binding was so weak. Of the three other mutants, Avm-Y33A displayed the largest alteration in affinity towards 2-iminobiotin (50-fold decrease), whereas Avm-Y33F and Avm-Y33H exhibited only minor changes in the dissociation constant (IV, Table 1). Interestingly, with all these three mutants only the dissociation rate was affected, whereas the association rate remained relatively unaffected despite the substitutions.

To test whether these mutations have any effect on the stability of the quaternary structure of avidin, a SDS-PAGE based thermal stability assay was executed (IV, Fig. 3). Of the four mutants, only Avm-Y33F exhibited stability characteristics similar to wild-type avidin, requiring temperatures over 50 °C in the absence of biotin and temperatures over 90 °C in the presence of biotin for denaturation. The three other avidin variants were clearly less stable than wild-type avidin or Avm-Y33F, dissociating partially or completely into monomers already at room temperature in the absence of biotin. The adding of biotin also stabilized the tetrameric structure of these three proteins as expected, but they still displayed diminished thermal stability when compared to that of wild-type avidin or Avm-Y33F.

In order to further characterize the stability properties of different mutants, their susceptibility to proteinase K digestion was tested (IV, Fig. 4). Avm-Y33F, Avm-Y33H and Avm-Y33Q all behaved similarly to wild-type avidin so that in the absence of biotin they were slowly degraded, whereas the addition of biotin rendered them resistant to any proteolysis by proteinase K. Interestingly, Avm-Y33A was shown to be substantially more vulnerable to digestion without biotin, whereas with biotin it was almost as stable as wild-type avidin or the other mutants.

Since Avm-Y33A, Avm-Y33H and Avm-Y33Q were found to be more or less monomeric already at room temperature in the presence of SDS (IV, Fig. 3) we decided to test the status of their quaternary structure in non-denaturing conditions with gel filtration FPLC. In all three different pH conditions (4, 7.2 and 11) used in this study, the three mutant proteins behaved similarly to wild-type avidin, forming stable tetramers both in the presence and in the absence of biotin.

6 DISCUSSION

The complex formed between vitamin biotin and chicken avidin is the tightest non-covalent interaction between a protein and its ligand found in the nature. Avidin is also one of the most stable proteins known, withstanding high temperatures, extremes of pH and high concentrations of denaturants without losing its functional structure (Green 1975). These unique properties of avidin together with the ability of biotin to be easily attached to many biological molecules have enabled its use in many different applications in the life sciences (Wilchek & Bayer 1990).

While the versatility of native avidin is impressive, protein engineering techniques provide new possibilities for creating avidin variants with enhanced properties that can be beneficial in certain applications. In attempting to improve the characteristics of avidin, we chose rational design as our strategy. Knowledge of the three-dimensional structure of avidin (Livnah et al. 1993, Pugliese et al.1993, Pugliese et al.1994) together with the sequence information from the avidin-related proteins (Keinänen et al. 1988, Keinänen et al. 1994, Ahlroth et al. 2000, Ahlroth et al. 2001), streptavidin (Chaiet et al.1963) and sea urchin fibropellins (Hunt & Parker 1989, Bisgrove et al.1991) have allowed intelligent design of mutants. This has considerably reduced the number of variants needed to produce and study to achieve the desired properties.

Using the above strategy we changed the charge properties of avidin by constructing a series of fully functional reduced-charge mutants (I). We then combined one of these mutants with a non-glycosylated avidin variant in order to improve the non-specific binding characteristics of avidin (II). We also introduced mutations on the different interfaces between avidin monomers in order to dismantle the avidin tetramer into smaller components (III).

Furthermore, avidin mutants with reduced biotin-binding affinity were also manufactured (IV).

6.1 Avidin mutants with reduced charge (I)

The goal of this study was to investigate whether the high positive charge of avidin can be reduced by carefully chosen point mutations without compromising the biotin-binding affinity or the high stability of the avidin tetramer. Consequently, a series of six avidin pI mutants was created with isoelectric points ranging from 9.4 to 4.7 (I, Table 1). In designing these mutations we turned to nature for guidance. Since streptavidin and most avidin-related proteins bind biotin with high affinity, we surveyed the respective positions of the various arginines and lysines of avidin in their sequences and made the substitutions accordingly. For example Arg-2 of avidin was exchanged to Ala and Lys-3 to Glu according to the sequence of streptavidin whereas Arg-59 was replaced with Ala, since in all AVR's this position is occupied by alanine. Where feasible, arginine residues were chosen for mutagenesis rather than lysines, since the terminal amino groups of the lysines are in many applications crucial for the derivatization of avidin.

Based on the optical biosensor data all the reduced charge mutants displayed biotin-binding characteristics comparable to those of native avidin. With all the mutants the binding to a biotin surface was extremely strong and irreversible and hence it was not possible to determine the actual dissociation constants with this method. Consequently, 2-iminobiotin, a biotin analog that binds avidin reversibly in a pH-dependent manner (Heney & Orr 1981), was used to further study the possible differences in the binding properties of the charge mutants. In the case of streptavidin, 2-iminobiotin has turned out to be a good analyst for the natural streptavidin-biotin interplay (Chilkoti et al. 1995), and most likely this is also the case with avidin. The dissociation constants of the different mutants exhibited some fluctuation (I, Table 1) but overall the binding to 2-iminobiotin was similar to that of native avidin. These results together with the irreversible binding to the biotin surface indicate that the mutations had little effect on the binding characteristics of the different mutant proteins. Regarding the minimal effect of the mutations on the biotin binding of avidin, it is important to note that according to the 3-D structure of the avidin tetramer, all the altered amino acids are located on the surface of the protein and have no major role either in binding of biotin or in stabilizing the quaternary structure (Livnah et al. 1993, Pugliese et al. 1993). In this respect, it has been shown that at least in the cases of lysozyme (Matthews 1995) and cutinase (Petersen et al. 1998), the mutations on the surface residues result only in minor changes in the overall 3-D structure of the protein. Recently, the same phenomenon was also demonstrated in the case of avidin, when Nardone and co-workers (1998) published a structure of an acidic avidin mutant with pI of

5.5, showing that the structures of the mutant and native avidin were almost identical.

Interesting results were obtained when the thermal stability of the different charge mutants was studied. Three of the six mutants, namely AvdpI9.4, AvdpI9.0 and AvdpI4.7, exhibited comparable dissociation profiles to that of wild-type avidin, requiring temperatures over 50 ° C or 90 ° C for denaturation, depending on the absence or the presence of biotin (I, Fig. 3). On the other hand, without biotin AvdpI7.9, AvdpI7.2 and AvdpI5.9 dissociated into monomers already at the room temperature when SDS was present. However, the addition of biotin stabilized these three mutants to such an extent that temperatures over 90 ° C were needed for these proteins to undergo denaturation (Fig. 9), as is the case with native avidin. AvdpI7.9, AvdpI7.2 and AvdpI5.9 all have mutations on both Lys-3 and Lys-9, suggesting that replacing either of these residues (or both) with negatively charged residue somehow weakens the tetrameric structure of avidin. However, AvdpI4.7 also bears these two mutations and yet still displays thermal stability characteristics similar to that of wild-type avidin. One potential explanation for this enigma is the possibility that the additional mutations in AvdpI4.7 (Arg-26-Ala and Arg.59-Ala) in someway compensate for the loss of stability attributed to these lysine replacements.

Bayer and co-workers (1996) have previously reported that at a low temperature wild-type avidin formed aggregates and failed to penetrate the separation gel in the SDS-PAGE analysis. The reason for this problem is attributed to the high positive charge of avidin, which presumably causes interaction with the negatively charged detergent. At higher temperatures, the aggregates dissociate, and the protein penetrates the gel as a monomer. It is therefore interesting to note that AvpI4.7 (I, Fig. 3) and all the other charge mutants, except AvpI9.4 and AvpI9.0, migrated under corresponding non-denaturing conditions in the separating gel as a tetramer when biotin was present. In this respect these reduced charge mutants could be useful in the electrophoretic analysis of avidin complexes with polybiotinylated proteins under native conditions, since they are normally difficult to characterize.

6.2 A non-glycosylated and acidic variant of avidin (II)

As previously discussed avidin and streptavidin are widely used molecular tools in biotechnological, diagnostic and therapeutic applications, collectively known as (strept)avidin-biotin technology (Wilchek & Bayer 1990). However, the positive charge and the presence of an oligosaccharide moiety in avidin have been hindrance to its use in many applications owing to the non-specific binding and high background levels of avidin. Consequently, streptavidin, a non-glycosylated and slightly acidic protein, has virtually replaced avidin in these applications despite the fact that avidin contains more lysines for

attachment of the probes, is more hydrophilic and is considerably cheaper than streptavidin. Furthermore, streptavidin is a highly immunogenic protein and it has its own non-specific binding problems due to the RYD peptide sequence, which resembles the integrin-binding motif RGD causing non-specific binding to cell surfaces (Alon et al. 1990, Alon et al. 1992).

In trying to amend the physicochemical properties of avidin, we have previously shown that using protein engineering the high pI of avidin can be reduced to 4.7 by replacing positively charged residues with neutral or acidic ones (I). In the present work we first mutated the glycosylation site of avidin in order to produce a non-glycosylated avidin variant and to study in detail whether the removal of the oligosaccharide side chain has any effect on the properties of avidin. The glycosylation site of avidin was abolished by replacing Asp-17 with Ile due to the fact that all AVR8s except AVR7 bear isoleucine on the corresponding location. Secondly, we combined this sugarless mutant with one of the previously described charge mutants (Avm-pI4.7) to further improve the non-specific binding characteristics of avidin. In fact, it has previously been demonstrated that an enzymatically deglycosylated and chemically modified neutral avidin (Neutra-Lite Avidin, Belovo chemicals, Belgium) is devoid of these non-specific binding problems and is in this way superior to native avidin in many applications (Bayer & Wilchek 1994). However, since the enzymatic and chemical procedures used in the preparation of this avidin derivative are often incomplete and lead to a mixture of products, it would be valuable to have a recombinant avidin mutant that is completely lacking sugar residues and has no positive charge.

As previously discussed, the mutations made to reduce the charge of avidin had only a nominal effect on the biotin-binding affinity of the charge mutants (I). This was also the case with non-glycosylated avidin variants ngAvm and ngAvm-pI4.7, since both of these proteins bound to the biotin surface in an irreversible fashion. Moreover, the kinetic parameters (both on and off rates) of these mutants for binding to 2-iminobiotin were similar to those of native avidin (II, Table 2) indicating that the removal of the oligosaccharide moiety has no effect on the biotin-binding ability of these proteins. This conclusion is further supported by the fact that bacterial streptavidin, which also binds biotin with extremely high affinity is also devoid of sugars.

When the thermal stability of the non-glycosylated mutants was studied, it was shown that ngAvm exhibited stability characteristics comparable to those of the native avidin both with and without biotin (II, Fig. 3). As previously shown, Avm-pI4.7 was also found to be as stable as native avidin (I, Fig. 3) whereas the combined mutant ngAvm-pI4.7 displayed somewhat lowered stability both in the absence and in the presence of biotin (II, Fig. 3). However, temperatures over 80 °C were still required for the dissociation of ngAvm-pI4.7 when biotin was bound, indicating that the stability of this variant was still high. Together with the affinity data these results support the earlier statements

that the sugar side-chain is not essential for the tight binding of biotin or the stability of the functional structure of avidin (Hiller et al. 1987, Bayer et al. 1995).

The results obtained from the proteinase K stability assay (II Fig. 4) showed that in the absence of biotin the non-glycosylated mutants, ngAvm and ngAvm-pI4.7 were degraded at a clearly faster rate than native avidin or Avm-pI4.7. In the presence of biotin, however, all four proteins behaved similarly, being fully resistant to any degradation by proteinase K. Ellison and co-workers (1996) have previously observed that the slow proteolysis of avidin in the absence of biotin is the result of the limited attack of the enzyme at the flexible loop between α -strands 3 and 4. However, when biotin is bound to avidin this loop becomes ordered and locks biotin into the binding site and renders avidin resistant to proteolysis. According to the 3D-structure (Livnah et al. 1993, Pugliese et al. 1993), the glycosylation site of avidin, Asn-17, is located close to this loop and therefore it is plausible that the oligosaccharide moiety creates some kind of steric hindrance that slows down the proteolysis, which would explain the faster degradation of the sugarless variants.

The non-specific binding of avidin and different mutant proteins to DNA and to four cell types was tested in a slot-blot assay (II, Fig. 5). As expected, wild-type avidin bound strongly to DNA due to its high positive charge. The same phenomenon was observed with ngAvm, which also has the high pI characterizing wild-type avidin. On the other hand, the two other mutants, Avm-pI4.7 and ngAvm-pI-4.7, which have a lowered pI exhibited no binding to DNA. Similar results were obtained regarding the non-specific binding to different cells i.e. wild-type avidin and ngAvm displayed strong binding whereas the variants with reduced charge showed little or no binding at all. These results further strengthen the earlier claims that the positive charge and the sugar moiety of avidin are the major factors entailing the non-specific binding and high background of avidin in some applications (Wilchek & Bayer 1990, Bayer & Wilchek 1994). Furthermore, these results are also in good agreement with the earlier observations that chemical neutralization and enzymatic deglycosylation of native avidin clearly improve the non-binding specific properties of avidin.

In the light of the results obtained, the non-glycosylated acidic mutant ngAvm-pI4.7 turned out to be a "winner" mutant. It still possessed the coveted features of native avidin, being able to bind biotin extremely tightly and having stable tetrameric structure while lacking the disadvantageous high pI and oligosaccharide moiety. Consequently, this avidin variant could be found useful in applications such as localization or separation studies, where non-specific binding and high background can cause problems. In addition, this mutant avidin may offer new possibilities for the use of avidin in affinity-based drug targeting for two reasons. Firstly, the high pI and the sugar side chain have been identified as major reasons for the rapid removal of avidin from the blood circulation (Kang et al. 1995, Rosebrough & Hartley 1996, Chinol et al. 1998) and ngAvm-pI4.7 could provide the solution to this problem. Secondly, while avidin is clearly less immunogenic than streptavidin, it can evoke immune

response when administered in repetitive doses (Chinol et al 1998). It may well be that ngAvm-pI4.7 could also be advantageous in this respect, since at least in the case of streptavidin the antibody response can be reduced by mutagenesis of surface residues (Meyer et al. 2001). In this regard, we have recently done some preliminary tests concerning the response of different avidin mutants to avidin antibodies and, indeed, it seems that the avidin antibodies used in this study (one polyclonal and two monoclonal) recognise ngAvm-pI4.7 poorly (unpublished results). It is also noteworthy that in a related study Nardone and co-workers (1998) reported that an acidic mutant of avidin (pI 5.5) exhibited distinct antigenic properties when compared to those of wild-type avidin. Therefore, it would be intriguing to thoroughly test the pharmacokinetic and immunogenic characteristics of ngAvm-pI4.7.

6.3 Monomeric avidins that undergo tetramerization upon biotin binding

As earlier discussed, there are three different monomer-monomer interactions in avidin which contribute to the rigidity of the quaternary structure (III, Fig. 1A). Of these three interfaces, 1-4 makes the largest contribution to the tetrameric structure and consists of several polar and hydrophobic interactions. In contrast, interfaces 1-2 and 1-3 are much more compact and contain only a small number of critical interactions (Livnah et al. 1993, Pugliese et al. 1993). We have previously reported that the interface 1-2 can be disassembled by replacing Trp-110 with lysine, resulting in dimeric avidin with reversible biotin-binding ability (Laitinen et al. 1999). In the current study our aim was to dismantle the avidin tetramer into monomers by changing selected residues from the interfaces 1-3 and 1-4 into alanines. Consequently, all the three hydrophobic residues from the interface 1-3 were altered (III, Fig. 1B) and the ensuing mutant was named Avm-3. From the interface 1-4 Asn-54 was first selected for mutagenesis (Avm-4a), since it forms an extensive network of hydrogen bonds between the monomers one and four (III, Fig.1D). Moreover, these two mutants were combined in Avm-[3,4a] to study the cumulative effects of these changes on the quaternary structure of avidin. In addition, a fourth variant Avm-[3,4b] was constructed which bears an additional mutation (Asn69Ala) for destabilizing the interface 1-4.

The FPLC results (III, Fig. 6) indicated that the mutations in Avm-3 and Avm-4a were not sufficient for the disassembly of the quaternary structure of avidin since both these proteins formed tetramers in the absence and in the presence of biotin. On the contrary, the combined mutants Avm-[3,4a] and Avm-[3,4b] were found to exist in monomeric state when biotin was excluded. However, when biotin was added a stable tetrameric structure was unexpectedly reintroduced. Similar results were obtained when the thermal stability of the different avidin mutants was studied (III, Fig.4). Without biotin,

Avm-3 and Avm-4a existed mostly in tetrameric form at room temperature even in the presence of SDS, whereas the combined mutants were completely monomeric. However, when biotin was introduced all the mutant proteins exhibited high stability characteristics similar to those of native avidin.

The results obtained from the FPLC and thermostability assay implied that the mutations constructed were successful in decreasing the stability of the quaternary structure of avidin. Although the changes made only in one interface (i.e. Avm-3 and Avm-4a) were not sufficient to break down the tetrameric structure as such when combined (Avm-[3,4a] and Avm-[3,4b]), they created monomeric avidin. This was only true, however, when biotin was excluded, since in the presence of biotin also these mutants formed stable tetramers. The reason for this peculiar behavior is most likely the additional interaction in the interface 1-2 upon biotin binding between Trp-110 (from the adjacent monomer) and biotin in the binding pocket. These results further strengthen our previous statements of the critical importance about the 1-2 interface, and especially Trp-110, for the stability of tetrameric avidin (Laitinen et al. 1999). Similar results have also been obtained with streptavidin, where mutation of the analogous Trp-120 into phenylalanine resulted in weakened intersubunit association together with reduced biotin binding affinity (Sano & Cantor 1995).

Of the four avidin variants only Avm-4a exhibited completely irreversible binding to biotin corresponding to that of native avidin whereas the other three showed some levels of reversibility ranging from 30 % to 45 %. However, when the binding to 2-iminobiotin was measured, both Avm-3 and Avm-4a displayed binding characteristics comparable to those of native avidin (III, Table 2). The actual kinetic parameters for the Avm-[3,4a] and Avm-[3,4b] binding to 2-iminobiotin could not be determined due to the complicated binding data caused by the monomer-tetramer shift upon binding. Nevertheless, these proteins also showed strong binding to 2-iminobiotin, as further proven by the fact that they were efficiently affinity purified using this biotin analog as a capturing ligand.

All four mutant variants were degraded at a substantially faster rate by proteinase K than native avidin in the absence of biotin. However, the introduction of biotin stabilized these proteins so that little or no degradation was observed (III, Fig. 5). These results together with our earlier observation that dimeric avidin is rapidly degraded both in the absence and in the presence of biotin (Laitinen et al. 1999) suggest that the tetrameric structure itself may not be sufficient to protect avidin from proteolytic digestion but that biotin binding is also required.

In light of these and previous results (Sano & Cantor 1995, Laitinen et al. 1999), it would seem that the high affinity for biotin and the extraordinary stability of avidin and streptavidin go hand in hand and that it is difficult to separate them. This conclusion is further strengthened by a recent study in which Qureshi and co-workers (2001) were producing streptavidin mutants with reduced biotin-binding affinity by changing some of the amino acids that

form hydrogen bonds with biotin and “accidentally” ended up with monomeric streptavidin. Furthermore, these results also indicate that (strept)avidin -barrel is itself a stable structure and does not require other subunits or biotin for proper folding. Finally, the biotin-induced monomer-tetramer transition described in this study could also provide new tools for analyzing protein-protein interactions. For example these avidin variants could be fused to different intracellular regulatory proteins, which could then be activated by oligomerization upon biotin binding.

6.4 Avidin mutants with lowered affinity for biotin (IV)

Although the extremely tight binding of biotin forms the bases of the (strept)avidin-biotin technology, in some applications, such as affinity chromatography or protein immobilization, the irreversible nature of the (strept)avidin-biotin complex can be a severe hindrance. This is due to the fact that extreme denaturing conditions are normally required for disrupting the (strept)avidin-biotin complex, which in turn usually leads to inactivation of the biotinylated molecule, thus rendering it unsuitable for subsequent use. In order to overcome this problem a monovalent avidin column has been developed in which it is possible to purify and elute biotinylated molecules in mild conditions with free biotin (Green & Toms 1973, Kohanski & Lane 1990). Unfortunately, this approach requires the treatment of immobilized avidin with strong denaturing agents for dissociation of the tetrameric structure, and such conditions are deleterious to both the carrier and the immobilized monomer. Both chemically and genetically modified versions of avidin and streptavidin with reversible binding to biotin have also been reported previously (Morag et al. 1996, Laitinen et al. 1999, Qureshi et al. 2001). The problem with the chemically modified nitro-avidin is that the nitration reaction of the tyrosine residue is not complete and therefore leads to a mixture of different end products. Furthermore, the genetically engineered avidin and streptavidin mutants with reversible binding characteristics described so far also display changes in their quaternary structure, being either dimeric or monomeric, and their affinity to biotin is only in the range of 10^6 - 10^8 M⁻¹. For these reasons it would be useful to have recombinant avidin or streptavidin variant that still continues to possess four stable, high-affinity binding sites for biotin but would allow binding to be reversed either with biotin or by changing the binding conditions.

On the bases of the X-ray crystal structure of avidin (Livnah et al. 1993, Pugliese et al. 1993, Pugliese et al. 1994) and streptavidin (Hendrickson et al. 1989, Weber et al. 1989) in complex with biotin, three structural motifs common to many high affinity binding proteins can be found: hydrophobic interactions between the ligand and the aromatic side chains in the binding pocket; ordering of flexible surface loops upon ligand binding and an extensive hydrogen

bonding network. It has been shown in previous studies both with avidin (Laitinen et al. 1999) and with streptavidin (Chilkoti et al. 1995, Sano et al. 1995) that substitution of the hydrophobic residues in the binding site can have substantial effects on both the biotin-binding affinity and stability of the quaternary structure. On the other hand, since individual interactions via H-bond are usually relatively weak, replacement of avidin residues forming hydrogen bonds with biotin might provide a possibility to fine-tune the affinity between avidin and biotin.

Consequently, Tyr-33 of avidin was chosen as our target for mutagenesis for several reasons. According to structural data this residue makes an important H-bond with the ureido oxygen of biotin, and it has been suggested that together with two other residues (Asn-12 and Ser-16) it forms a hydrogen bonding network that focuses complementary electrostatic interactions with the polarized ureido oxyanion (Fig. 1) (Weber et al. 1992). Furthermore, it has been shown that by nitration of Tyr-33 in avidin (or the corresponding residue Tyr-43 in streptavidin) reversible biotin binding can be achieved (Morag et al. 1996). In addition, chemical modification of this tyrosine residue at the hydroxyl group with p-nitrobenzenesulphonyl fluoride eliminates the biotin-binding ability completely (Gitlin et al. 1990).

In this regard it was somewhat surprising that substitution of this Tyr-33 with Phe had hardly any effect on the biotin-binding affinity: Avm-Y33F displayed only a 4-fold decrease in binding to 2-iminobiotin (IV, Table 1) and exhibited completely irreversible binding to biotin (IV, Fig. 2) similar to the tenacious binding by wild-type avidin. These findings strongly indicate that the hydrogen bond formed between the hydroxyl group of Tyr-33 and the ureido oxygen of biotin is not as essential as previously thought. This seems to be also the case with streptavidin, since Klumb and co-workers have obtained similar results when studying the energetics of the hydrogen bonding between the ureido oxygen of biotin and streptavidin (Klumb et al. 1998). In addition, it has been proposed that the reason for the pH-dependent binding of 2-iminobiotin to avidin is based on the fact that at a higher pH the guanido group of 2-iminobiotin is able to make the H-bond with hydroxyl group of Tyr-33, whereas at lower pH the guanido group takes up an extra hydrogen and cannot form the bond (Hoffman et al. 1980, Orr 1981). However, the results obtained in this study suggest that some other mechanisms may be involved as well, since abolishing this H-bond alone does not seem to be sufficient for the release of biotin from the binding site. In this regard, Katz has recently proposed that with streptavidin at a low pH the loss of the H-bond between Asn-23 and 2-iminobiotin nitrogen together with the fact that Ser-27 is forced to accept two hydrogen bonds rather than the more favorable arrangement of accepting one and donating one also contribute to the decreased affinity of this ligand (Katz 1997). Similar factors might also explain the reduced affinity of 2-iminobiotin towards avidin at a low pH.

Since Avm-Y33F was shown to bind biotin in an irreversible fashion, three other mutants were designed in order to further decrease the binding affinity of

avidin. With Avm-Y33A we wanted to decimate both the H-bonding of the hydroxyl group and the hydrophobic interaction between the aromatic ring structure of Tyr/Phe, whereas in Avm-Y33Q the hydrophobic interaction was also abolished but the hydrogen bonding would be possible through the nitrogen of the amide group. When the binding characteristics of Avm-Y33A were studied it was shown that this substitution resulted in a clear reduction in affinity towards 2-iminobiotin (IV, Table 1). However, the binding to the biotin surface was still practically irreversible (IV, Fig. 2). With Avm-Y33Q we were unable to measure the kinetic parameters of binding to 2-iminobiotin with the biosensor since it displayed such a low affinity. This is in good agreement with the poor recovery of this protein from the affinity purification. When the binding to the biotin surface was measured, Avm-Y33Q exhibited a substantially lower association rate than has been reported for native avidin. However, the dissociation of this protein from the biotin surface was so slow that we were unable to measure the off-rate for this binding. Nevertheless, since the detection limit of our optical biosensor for the dissociation rate is around $1 \times 10^{-5} \text{ s}^{-1}$, it can be estimated that the maximum K_d for the binding of Avm-Y33Q to biotin is around 10^{-10} M .

The rationale behind our fourth mutant Avm-Y33H was two-fold: Firstly, two of the sea urchin fibropellins (Hursh et al. 1987, Hunt et al. 1989) have histidine residue in the position analogous to Tyr-33 of avidin in their avidin-like domain. Secondly, the presence of His in this critical position in the binding pocket of avidin might lead to pH-dependent changes in the affinity towards biotin due to the deprotonation of the imidazole ring of histidine at higher pH. Of the four mutants reported in this study, Avm-Y33H was the most successful in respect of the reversibility of the biotin binding. Furthermore, this reversibility was clearly pH-dependent, since at a neutral pH (or lower) Avm-Y33H bound irreversibly to the biotin surface, whereas at elevated pH about 50% of the protein could be released with free biotin (IV, Fig. 2). Interestingly, the reversibility of the nitro-avidin in these binding conditions was approximately 65%, indicating that the reversibility of Avm-Y33H was reasonably good. The likely reason for the behavior of Avm-Y33H is the fact that at a low pH the His residue has a positive charge, and it interacts with the carbonyl group of the biotin, whereas at a high pH the imidazole ring of His is deprotonated, leading to loss of this interaction, and the binding is partly reversed (IV, Fig. 5). However, in the case of 2-iminobiotin the opposite situation holds, since at a low pH the guanido group of 2-iminobiotin also has positive charge, resulting in repulsion with the positively charged imidazole ring and hence no binding. At a higher pH both these positively charged groups are deprotonated, leading to disappearance of the repulsion.

Interestingly with Avm-Y33F, Avm-Y33A and Avm-Y33H, only the off-rates were affected when binding to 2-iminobiotin was tested, whereas the on-rates remained relatively unchanged (IV, Table 1). These results indicate that replacing Tyr-33 with these amino acids does not seem to result in notable alterations in the shape of the biotin-binding pocket of avidin. In this regard it is

interesting to note that the biotin-binding pocket in avidin seems to be constructed precisely to fit biotin and that in the absence of biotin the binding site contains five structured water molecules that emulate the structure of biotin. Indeed, this shape complementarity is thought to be one of the primary reasons for the extraordinarily high affinity that avidin exhibits towards biotin, since no conformational changes requiring energy are needed upon ligand binding (Wilchek & Bayer 1999). On the other hand, the substitution of the Tyr-33 with glutamine resulted in clearly slower binding to the biotin surface suggesting that the longer side chain of Gln may somehow distort the delicate geometry of the binding pocket.

Although Tyr-33 is located inside the β -barrel relatively distant from the monomer-monomer interfaces, its replacement with Ala, His or Gln resulted in a substantial reduction in the strength of the avidin tetramer in the thermostability assay both in the absence and in the presence of biotin (IV, Fig. 3). However, the in FPLC gel filtration column all the Tyr-mutants migrated in the same way as wild-type avidin, indicating that under non-denaturing conditions at room temperature these proteins form stable tetramers. Nevertheless, substituting the Tyr-residue with these three amino acids clearly somehow leads to an impaired quaternary structure despite the seemingly irrelevant position of Tyr-33 to the subunit-subunit interactions. A similar decline in the stability of the avidin tetramer was also observed with nitro-avidin (Morag et al. 1996) and it has also been reported recently that in the case of streptavidin mutagenesis of three amino acids (Ser-45, Thr-90, Asp-128) that form H-bonds with biotin result in completely monomeric protein (Qureshi et al. 2001). This study together with our earlier results from the dimeric/monomeric avidin variants (Laitinen et al. 1999, III) strengthens our previous speculation that the exceptional stability and the high ligand binding affinity of avidin are two sides of the same coin, and that it is difficult to separate them. In this respect additional structural studies regarding these Tyr-mutants and the dimeric/monomeric forms of avidin might shed more light on the formation and stability of the avidin tetramer. Furthermore, we are currently continuing our work with Avm-Y33H by introducing additional substitutions to the binding site in order to produce a tetrameric avidin variant that displays high affinity to biotin but is completely reversible under mild conditions.

7 CONCLUSIONS

The study presented in this dissertation is part of a larger project with the aim to understand the molecular bases of the extremely tight avidin-biotin complex and the factors affecting the tetrameric association and stability of avidin. Furthermore, the obtained knowledge can be used to create new tools for the applications of (strept)avidin-biotin technology by modifying certain properties of avidin. The main conclusions of this study are:

1. The charge properties (i.e. the high pI) of avidin can be modified by protein engineering without significantly disturbing the biotin-binding activity or the stability of the avidin tetramer.
2. The oligosaccharide moiety of avidin is not essential for biotin binding or for maintaining the high thermostability properties of native avidin.
3. A non-glycosylated and acidic avidin variant, ngAvmA-pI4.7, displayed substantially improved non-specific binding characteristics when compared to native avidin, suggesting that it could be useful in applications, such as localization and separation studies, where high background can be a problem.
4. It was possible to dismantle the avidin tetramer into monomers by mutating selected residues in interfaces one-to-three and one-to-four. However, upon biotin binding these monomeric variants reorganized into tetramers, thereby demonstrating the importance of the 1-2 interface and, especially, of Trp-110 (from adjacent subunit), for the stability of the avidin tetramer.

5. The hydrogen bond formed by the hydroxyl group of Tyr-33 with the ureido oxygen of biotin is not as essential for the tight binding of biotin as previously thought. In addition, by substituting Tyr-33 with His we were able to create an avidin variant that exhibited clear pH-dependence in its biotin binding affinity.
6. In summary, the results from this study indicate that avidin can withstand mutations, even radical ones, in the amino acid sequence as long as the changes are restricted to surface residues. On the other hand, the high ligand-binding affinity and the extreme stability of avidin tetramer seem to go hand in hand and it is very difficult to modify either one without affecting the other.

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YHTEENVETO (Résumé in Finnish)

Avidiinin varauksen, biotiininsitomisen sekä oligomerisaation muokkaaminen: uusia työkaluja avidiini-biotiiniteknologiaan

Avidiini on neljästä samanlaisesta alayksiköstä koostuva proteiini, jota esiintyy pieninä määrinä kananmunan valkuaisessa. Avidiinilla on useita mielenkiintoisia ominaisuuksia, joiden ansiosta tämä proteiini on ollut tutkijoiden kiinnostuksen kohteena jo useita vuosikymmeniä. Merkittävin avidiinin piirre on sen kyky sitoa vesiliukoista vitamiinia, biotiinia. Avidiinin ja biotiinin välinen sidos on erittäin voimakas, ja sitä onkin opittu käyttämään hyväksi lukuisissa erilaisissa sovellutuksissa biotieteiden ja lääketieteen eri aloilla. Avidiini on myös äärimmäisen stabiili proteiini, joka kestää erilaisia ääriolosuhteita, kuten korkea tai matala pH, korkea lämpötila tai erilaisia denaturoivia liuoksia. Esimerkiksi sidottuaan biotiinia, avidiini säilyttää toimivan rakenteensa täydellisesti normaalissa veden kiehumislämpötilassa.

Viime vuosien aikana ovat erilaiset proteiinien muokkaustekniikat kehittyneet vauhdilla. Näiden menetelmien avulla voidaan olemassa olevien proteiinien ominaispiirteitä muunnella tai jopa luoda niille kokonaan uudentyyppisiä funktioita. Väitöskirjatyössäni pyrittiin muokkaamaan avidiinin erilaisia ominaisuuksia kohdennetun mutageneesin avulla. Tavoitteena oli toisaalta tutkia millaisia muutoksia avidiinin aminohapposekvenssiin voidaan tehdä ilman että se menettää toiminnallisen rakenteensa ja toisaalta myös kehittää uusia paranneltuja versioita avidiinista erilaisia sovellutuksia varten. Halutut mutaatiot avidiinin aminohappojärjestykseen suunniteltiin hyväksikäyttäen avidiinin tunnettua kolmiulotteista rakennetta sekä sekvenssi-informaatiota avidiinia muistuttavista proteiineista.

Väitöskirjan ensimmäisessä osatyössä tutkittiin, voidaanko avidiinin varausominaisuuksia muokata ilman että biotiinin sitomiskyky tai kestävyys heikkenevät. Tätä varten suunniteltiin sarja varausmutantteja, joissa avidiinin korkeaa isoelektristä pistettä laskettiin asteittain. Kaikki näin saadut avidiini-muodot sitoivat biotiinia tehokkaasti, ja useimmissa tapauksissa myös kestävyysominaisuudet säilyivät ennallaan. Toisessa osatyössä tarkoituksena oli tuottaa avidiinia ilman sokerisivuketjua poistamalla sen luontainen N-glykosylaatiosekvenssi. Lisäksi tämä sokeriton avidiini-mutantti yhdistettiin yhteen edellä mainituista varausmutanteista avidiinin bioteknologisten ominaisuuksien parantamiseksi. Sokerisivuketjun poistamisella avidiinista ei havaittu olevan merkittävää vaikutusta biotiinin sitoutumiseen tai proteiinin kestävyysominaisuuksiin. Sen sijaan yhdistelmämutantti (ei sokeria, matala isoelektrinen piste) osoittautui ylivertaiseksi tavalliseen avidiinin verrattuna epäspesifisen sitoutumisen puuttumisen takia, ja tästä syystä se sopii käytettäväksi erityisesti sellaisissa

bioteknisissä ja lääketieteellisissä sovellutuksissa, joissa normaalin avidiinin käyttö on hankalaa korkean taustasitoutumisen vuoksi.

Kolmannessa osatyössä oli tavoitteena hallitusti hajottaa avidiini tetrameeri biotiinia sitoviksi monomeereiksi vaihtamalla kolmiulotteisen rakenteen perusteella huolellisesti valittuja alayksiköiden välisiin sidoksiin osallistuvia aminohappoja alaniineiksi. Tuloksena saatiin kaksi monomeeristä avidiinimutanttia, jotka hieman yllättäen kuitenkin järjestyivät uudelleen tetrameereiksi sidottuaan biotiinia. Neljännessä osatyössä pyrittiin muokkaamaan avidiinin biotiinin sitomiskykyä palautuvampaan suuntaan, jolloin esimerkiksi käytettäessä avidiinia puhdistuskahvana se saataisiin helpommin irtoamaan biotiinia sisältävästä pylväästä. Tätä tarkoitusta varten mutatoitiin avidiinin Tyr-33 aminohappoa. Mutaatioiden seurauksena saatiin avidiinin affiniteettia biotiinia kohtaan pudotettua, mutta näillä muutoksilla oli myös vaikutusta avidiinitetrameerin stabilisuuteen. Mielenkiintoisin näistä tyrosiinimutanteista oli AvmY33H, jossa tämä aminohappo oli korvattu histidiinillä. Johtuen histidiini-aminohapon fysikaalisista ominaisuuksista pH vaikutti voimakkaasti tämän avidiinimutantin kykyyn sitoa biotiinia.

Kokonaisuutena väitöskirjatutkimus osoitti, että avidiinin eri ominaisuuksia voidaan muokata kohdennettujen aminohappomuutosten avulla ja että avidiinin rakenne kestää näitä muutoksia hyvin, jos vaihdetut aminohapot sijaitsevat proteiinin pinnalla. Toisaalta kuitenkin näyttäisi siltä, että tiukka biotiinin sitominen ja avidiinin kestävyys kulkevat käsi kädessä ja että on vaikeaa muokata jompaa kumpaa ominaisuutta vaikuttamatta myös toiseen.

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