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Review

Genetically engineered avidins and streptavidins

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Abstract. Chicken avidin and bacterial streptavidin, (strept)avidin, are proteins widely utilized in a number of applications in life science, ranging from purification and labeling techniques to diagnostics, and from targeted drug delivery to nanotechnology. (Strept)avidin-biotin technology relies on the extremely tight and specific affinity between (strept)avidin and biotin (dissociation constant, $K_d \approx 10^{-14}$–10^{-16} M). (Strept)avidins are also exceptionally stable proteins. To study their ligand binding and stability characteristics, the two proteins have been extensively modified both chemically and genetically. There are excellent accounts of this technology and chemically modified (strept)avidins, but no comprehensive reviews exist concerning genetically engineered (strept)avidins. To fill this gap, we here go through the genetically engineered (strept)avidins, summarizing how these constructs were designed and how they have improved our understanding of the structural and functional characteristics of these proteins, and the benefits they have provided for (strept)avidin-biotin technology.

Keywords. Affinity, avidin, bionanotechnology, biotin, mutagenesis, protein engineering, streptavidin.

Introduction

If one wishes to alter chosen properties of any protein, there are basically two alternative possibilities. The conventional approach is to chemically modify the target protein. Several residue-specific chemicals are available for this purpose (reviewed by Lundblad and Bradshaw [1]). Another, more sophisticated way to specifically tune selected protein residue(s) is the rational or random mutagenesis of the DNA encoding the target protein, then producing the mutant protein form by one of the established heterologous expression systems. Both rationales have their own benefits and drawbacks.

Chemical modifications are restricted by the starting materials, whose intrinsic properties may hinder certain kinds of modification, or else the desired modification cannot usually be targeted specifically to single amino acid residues without modifying other chemically similar groups in the protein. On the other hand, by chemical modifications it is possible to create novel characteristics not attainable by other methods.

In comparison, mutagenesis not only allows the desired changes to be made in the fundamental building blocks of proteins, *i.e.* individual amino acid residues of the target, but also makes it possible to introduce more radical changes in its structure and topology. For example, new residues can be inserted, parts of the protein can be deleted and the order of the residues in the polypeptide chain can be altered *via* circular permutation. In addition, production of mutant forms of proteins can lead to better-quality products as compared with chemical modifications, as every molecule is translated similarly according to the mutagenized sequence. The main restriction in this strategy is that it relies on the use of 21 naturally occur-

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ring amino acids (20 amino acids coded by regular genetic code and selenocysteine coded by UGA stop codon and SElenoCysteine Insertion Sequence [SECIS element] [2]), although recently successful incorporations of non-natural amino acids to proteins *via* translation have been reported (reviewed in [3]). Naturally, the gene or cDNA, either natural or artificial, as well as the appropriate production system are required for a successful outcome.

In some cases combination of these two strategies may be required to obtain the desired result. A special case, which does not clearly belong to either of the above-described categories, is enzymatic treatment, which can be used, for example, to alter/remove post-translational modifications or to digest the target protein to smaller fragments.

Chicken avidin, originally isolated from egg white, as well as streptavidin, secreted by *Streptomyces* bacteria, are structurally [4, 5] and functionally [6, 7] analogous proteins, famous for their extremely high affinity for the small water-soluble vitamin, biotin ($K_d \approx 10^{-14}$–10^{-16} M). Avidin and streptavidin are a remarkable twosome that offers the scientist an almost ideal opportunity to explore the diverse properties of these proteins. They exhibit many features that single them out from the protein population. In addition to their exceptional ligand-binding properties, they are also particularly stable against heat [8], denaturants [9, 10] and low or high pHs [6], and, furthermore, they show great resistance even to the activity of number of proteolytic enzymes [6, 11]. This high-affinity protein-ligand interaction and the stability properties have aroused the attention of the scientific community mainly for two reasons. First, the practical impulse to such great interest derives from the possibility to utilize (strept)avidin-biotin in various applications, these currently including multiple fields of life sciences across a range of biochemical, pharmaceutical and biophysical applications [12, 13]. The second reason for the interest has been the revelation of the molecular details of the properties mentioned, *i.e.* the (strept)avidin-biotin pair serves as an interesting model system of a highly stable oligomeric protein displaying extremely high affinity toward a small ligand. The nature of the interaction is exceptional due not only to the affinity as such but also in the sense of binding energy per atom [14].

These factors explain the plethora of studies describing different chemical modifications and genetically engineered forms of avidin and streptavidin. Firstly, from the viewpoint of applications, one outcome has been regulation of the physicochemical and biotin-binding properties of (strept)avidin both to broaden the spectrum of their potential applications and conditions of use and to overcome some of the drawbacks inherent in the (strept)avidin-biotin system. They include, for instance, irreversibility of interaction and aggregation tendency caused by tetramerization in some applications, and non-optimal pharmacokinetics in applications *in vivo*. Secondly, a great many modification studies have been conducted to elucidate the different aspects of high-affinity ligand binding and the structural stability properties of (strept)avidins. In many cases, purely scientific objectives have produced new protein forms of value also in applications, and, *vice versa*, many modifications that have a basis in improving the applicative properties of (strept)avidin have led to new insights into structurally and functionally important aspects of these two proteins.

Structural properties of (strept)avidin

Both proteins, avidin and streptavidin, are secreted in their natural hosts [7]. In the case of avidin its signal sequence is cleaved off during the secretion process, whereas, in the case of streptavidin, the final product, so-called core streptavidin, is processed from both N and C termini (Fig. 1). In spite of their functional similarities, the primary structure of avidin and streptavidin is only moderately conserved with an amino acid similarity and identity percentage of 41% and 30%, respectively [15] (Fig. 1). Despite this, they have secondary, tertiary and quaternary structures almost identical to each other. Subunits of (strept)avidins are formed from eight antiparallel β-strands and their interconnecting loops [4, 5, 16, 17]. Most of the differences in primary structure are located in the loop regions of the tertiary structure. They fold as a classical antiparallel β-barrel (Fig. 2a) and their final quaternary structures are formed as a combined unit of the four identical barrels (Fig. 2b).

The biotin-binding site of (strept)avidin is located at one end of each β-barrel subunit (Fig. 2). Each tetrameric (strept)avidin molecule can therefore bind four biotin molecules [6]. As can be seen in Fig. 1, the biotin-binding residues of avidin and streptavidin are much better conserved than other residues [5], and there are also more biotin-binding contact amino acid residues in avidin than in streptavidin (Fig. 3). This may partially explain why the affinity between avidin and biotin is even tighter than that between streptavidin and biotin [18]. Both proteins have two main motifs for forming a biotin-binding site or pocket: first, the presence of hydrophilic contact residues forming hydrogen bonds with biotin, and second, the presence of several aromatic residues creating the hydrophobic environment required for the hydrophobic ligand. Especially the ureido-ring of biotin is efficiently hydrogen-bonded at the (strept)avidin-binding site [4, 5].

Even these multiple contacts cannot, however, completely explain the extreme affinity (strept)avidin exhibits to biotin. After the biotin binding has taken place, some modulations in their tertiary and quaternary structures can be seen [19, 20]. The conformation of the loop between β-strands three and four becomes defined in X-ray crystallography, and it can thus be considered as a

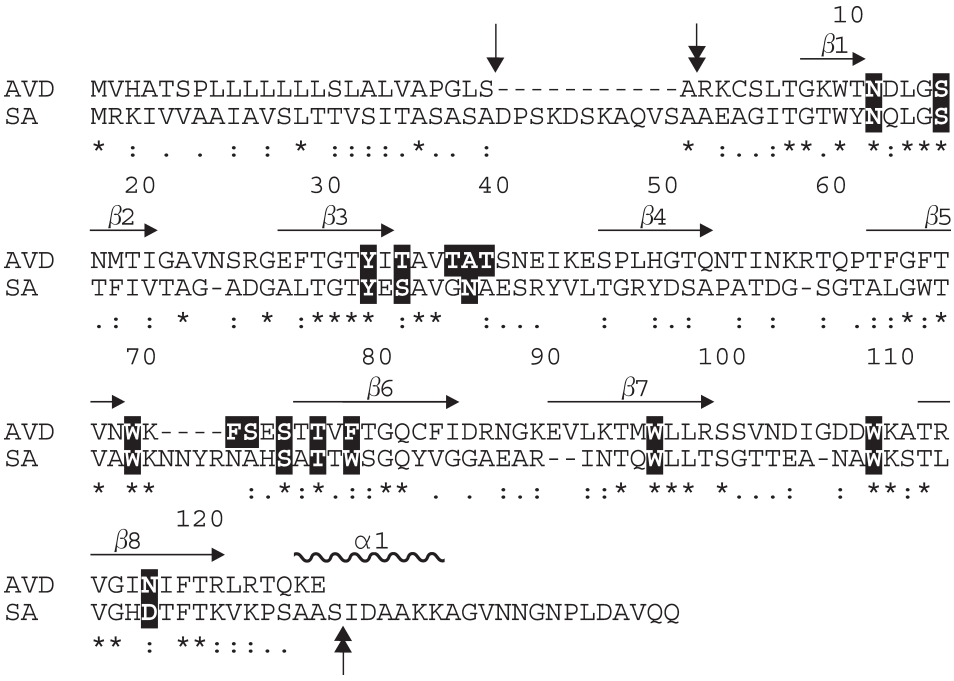

Figure 1. Alignment of avidin and streptavidin. The secondary structure elements are indicated by arrows. The secondary structure shown is according to avidin [5] except the C-terminal helix, which is present only in full-length streptavidin [22]. Residues forming direct contacts with biotin are shown against a black background. The cleaving site of the signal sequences both in avidin and streptavidin is indicated by vertical arrow. The experimentally determined most common cleavage sites of streptavidin polypeptide are shown by double-headed arrows (core streptavidin) [138]. The numbering follows the mature avidin sequence. Conserved residues are indicated with an asterisk below the alignment, strongly similar amino acid residues are indicated with a colon and weaker group similarity is indicated with a full stop.

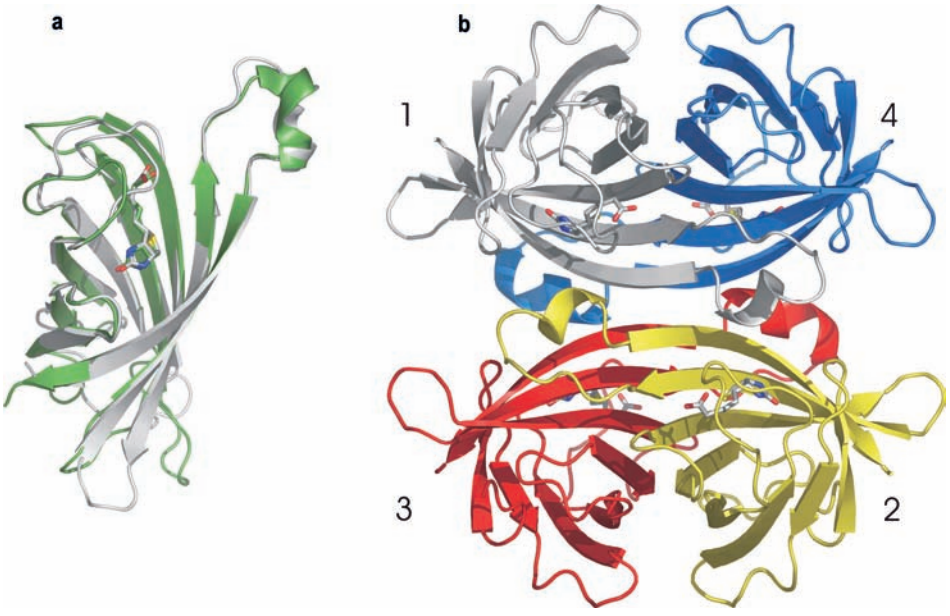

Figure 2. (*a*) Superimposed structure of avidin (gray, PDB 2avi [5]) and streptavidin (green, PDB 1mk5) barrels determined by X-ray crystallography. The secondary structure elements are indicated in cartoon presentation and bound ligand, d-biotin, is shown in sticks. (*b*) Avidin tetramer (PDB code 2avi [5]). The secondary structure elements are indicated in cartoon presentation and bound ligands, four d-biotin molecules, are shown in sticks. Subunits are numbered according to [5]. The figures as well as the following structural presentations were generated using the PyMOL program [139]

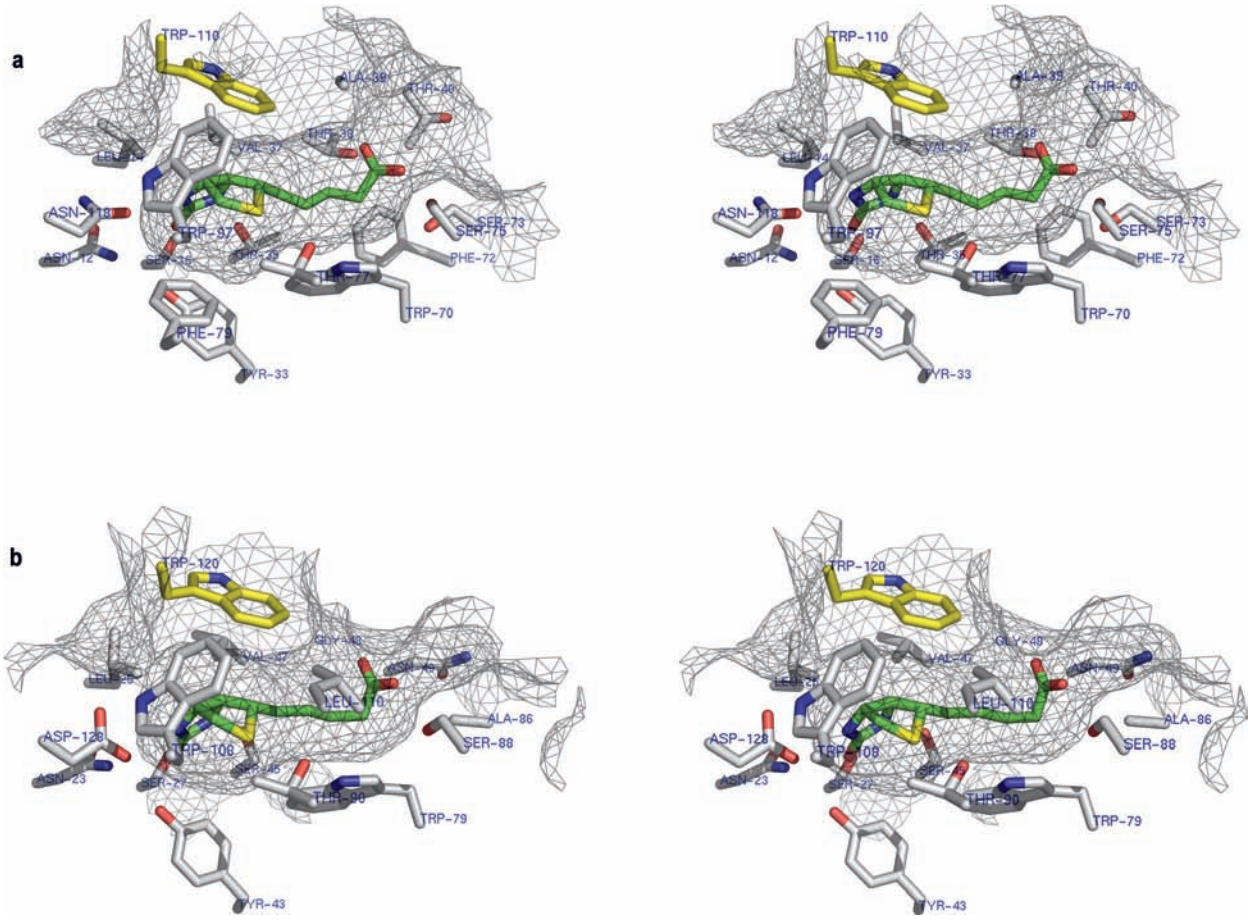

Figure 3. The shape of the biotin-binding site in (*a*) avidin (PDB 2avi [5]) and (*b*) streptavidin (PDB 1mk5). The ligand-binding cavity in the protein within 6 Å from biotin is visualized by gray mesh and the side chains of residues within 4 Å from the bound biotin are shown in sticks (biotin in green) and labeled. The Trp110/Trp120 residue from neighboring subunit is shown in yellow.

lid enclosing biotin in the barrel [4, 5]. Furthermore, the biotin binding has been found to slow down deuterium-hydrogen exchange in large parts of the protein molecule, indicating tighter packaging of the protein in the presence of the ligand [20]. The last factor contributing to the tight binding is the shape complementarity of the binding pocket with the ligand (Fig. 3). In the absence of bound biotin, five water molecules mimic a biotin molecule in the apoproteins [4, 5].

In the context of ligand binding, there is a functional interplay between the subunits, which takes place between subunits one and two and also between subunits three and four (numbered according to [5], see Fig. 2b, 3, 4). The major agent in this interplay is the conserved Trp110 of avidin or Trp120 in streptavidin, which is located in the loop between β-strand seven and eight. It forms part of the biotin-binding pocket of the neighboring subunit and *vice versa*, and therefore, subunits one and two (three and four) can be considered as a functional dimer. As structural entities, both the avidin and the streptavidin tetramer can be considered essentially a dimer of two kinds of dimers. Structural dimers are formed between monomers one and four (or two and three) (Fig. 4) in both proteins. The interface, comprising only three amino acid residues from subunits one and three (two and four), forms together with the functional dimer subunit pair a combined dimer-dimer interface. Notwithstanding its small size, the one-three interface is important for the oligomeric stability of (strept)avidin, as will be discussed in later sections.

In native preparations streptavidin exists as heterogeneous products [21]. These were shown to be differently processed polypeptides having both N-terminal and C-terminal extensions as compared with the fully mature core streptavidin (Fig. 1). Especially the cleavage of the C-terminal extension of streptavidin is important for its high-affinity biotin binding. The structural rationale for this observation was recently explored by Le Trong et al. [22], who solved the structure of the full-length streptavidin for the first time. According to this crystallographic structure, the C-terminal extension of 20 residues in length formed a defined loop on the surface of the streptavidin tetramer, and residues 150–153 bound to the

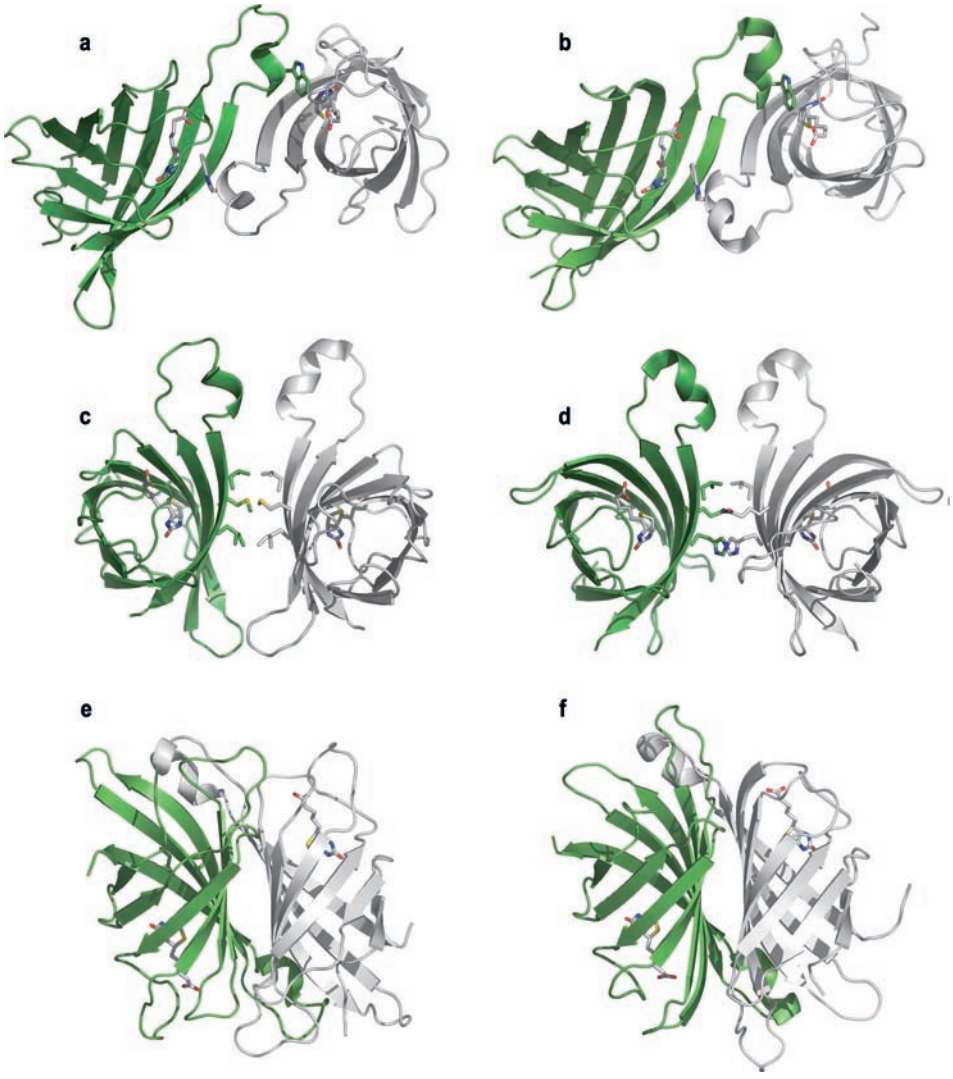

Figure 4. Interfaces between avidin and streptavidin subunits. (*a*) The interface between subunits one and two in avidin (PDB code 2avi) and (*b*) in streptavidin (PDB code 1mk5). Side chains of residues Trp110 and Trp120, respectively, are shown in sticks. (*c*) The interface between subunits one and three of avidin and (*d*) of streptavidin. Three central residues making contact over the interface are shown in sticks. The main chain oxygen and nitrogen atoms are removed for clarity (*c*, *d*). (*e*) The interface between subunits one and four in avidin and (*f*) in streptavidin.

biotin-binding site competing possibly with biotinylated compounds. This structure is flexible enough to dislodge from the binding site, allowing binding of small ligands, although it may form a steric hindrance for larger biotin complexes.

The essential parts for the production of avidin and streptavidin in heterologous expression systems, the full-length cDNA for avidin and the gene for streptavidin, were cloned in 1987 [23] and in 1986 [24], respectively. Since then different expression systems for (strept)avidin have been described. The production of recombinant streptavidin has been most often reported in bacteria, especially in gram-negative *Escherichia coli*. The use of cytoplasmic *E. coli* expression systems leads mainly to an accumulation of streptavidin as inclusion bodies, thus requiring renaturation and further downstream steps to obtain the active protein [25]. Alternatively, streptavidin has been produced in soluble form within the periplasmic space of *E. coli,* employing signal peptide from *E. coli* OmpA protein [26], and recently also within the cytoplasm of baculovirus-infected *Spodoptera frugiperda* insect cells [15] as well as in transgenic tobacco and apple [27]. In contrast to previous work, where streptavidin has always been produced in a core form, a recent study shows the production of mutagenized soluble streptavidin in the cytoplasm of *E. coli* a in full-length form [28]. In addition, for the production of soluble streptavidin, an efficient expression system in gram-positive *Bacillus subtilis* has been developed [29]. Chicken avidin, being a eukaryotic protein, has proved even more difficult than streptavidin

to produce in bacteria. Trials involving cytoplasmic expression in *E. coli* have produced low yields of the soluble protein or avidin aggregated as inclusion bodies [30]. In contrast, successful production with reasonable yields was achieved using eukaryotic production systems such as baculovirus-infected insect cells [31], *Pichia pastoris* [32] and in transgenic plants maize [33], tobacco [34] and apple [27]. Recently, successful production of active avidin in the periplasmic space of *E. coli* has also been reported [35]. The expression was achieved by replacing the original avidin signal peptide with a bacterial signal peptide from *Bordetella avium* OmpA.

Since the solution of the three-dimensional (3D) structures of avidin and streptavidin in the late 1980s [4, 16] and early 1990s [5, 17], respectively, and by reason of the vast amount of knowledge accrued regarding the functional characteristics of these proteins, most mutagenesis studies have been conducted on a rational basis. Some random or semi-random mutagenesis approaches have also, however, been used for (strept)avidin. The first studies demonstrating the power of mutagenesis approach were conducted in the case of streptavidin by Stayton and Cantor and their groups [36, 37] in the mid 1990s. The same was done with avidin by us a couple of years later in collaboration with Bayer and Wilchek from Israel [38], and separately by Arosio and his coworkers [38, 39].

In the following sections the individual cases of the different mutagenic modifications of avidin and streptavidin are addressed. We show how these studies have shed light on our fundamental knowledge of their functional and structural characteristics, and improved and/or modulated their practical properties in applications.

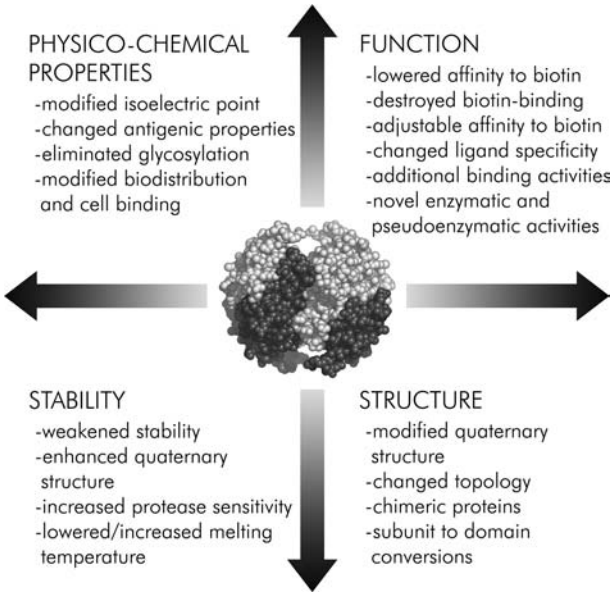

Figure 5. The outlines of (strept)avidin engineering. The three-dimensional structure of avidin is shown in CPK model (PDB 2avi).

Genetically engineered (strept)avidins

The modifications conducted are categorized in the following chapters as binding-site mutants, interface mutants, topology modifications, chimeric (strept)avidins, enzymatic and pseudoenzymatic (strept)avidin mutants and miscellaneous modifications (Fig. 5). Some modifications have effects on phenomena coincidently fitting two or more of these categories, and every case has thus been placed in the class that best describes its consequences. One important element, (strept)avidin fusion proteins, is not discussed here, the reason being that this review is clearly focused on site-directed mutagenesis studies or protein engineering, and that (strept)avidin fusion systems have been reviewed elsewhere [40–46].

Binding-site mutants

The biotin-binding site of (strept)avidin (Fig. 1, 3) has been one of the major targets of site-directed mutagenesis. The wild-type (wt) binding site displays a virtually perfect fit with biotin (Fig. 3), and mutants with reduced affinity can therefore be expected upon the introduction of almost any kind of mutations in the area [47]. Although most of the applications of (strept)avidin-biotin technology are based on the extreme affinity between (strept)avidin and biotin, mutants with reduced affinity and reversible binding characteristics would be particularly useful, for example, in separation techniques where biotin or the (strept)avidin mutant is coupled to a solid matrix. Lower avidin-ligand affinity can, however, also be achieved using biotin derivatives, for example, 2-iminobiotin. The interaction of (strept)avidin with 2-iminobiotin is pH dependent, and the complex can be dissociated at a mildly acidic pH [48].

The significance of the hydrophobic lining of the biotin-binding pocket has been studied by constructing mutant streptavidins where Trp79, Trp108 and Trp120 were separately substituted with alanine or phenylalanine. These mutants did, indeed, alter biotin and 2-iminobiotin binding properties [36]. The conservative phenylalanine mutants W79F and W108F showed slightly reduced affinity toward 2-iminobiotin, whereas the affinity of the W120F mutant was two orders of magnitude weaker than that of wt streptavidin (Table 1). All the alanine mutants showed markedly (four to six orders of magnitude) reduced 2-iminobiotin affinity values. The estimated biotin K_d values were 2.3×10^{-8} M for W79A, 1.2×10^{-7} M for W120A and $< 2 \times 10^{-10}$ M for W120F. Another alanine mutant, W108A, was unstable in the assay conditions. The assay could not differentiate the high affinities displayed by W79F and W108F from that of wt streptavidin. Interestingly, the K_d value of the W120F mutant with biotin

in an independent study was 1×10^{-8}–3×10^{-8} M; moreover, the binding was found to be rapidly reversible, since bound biotin was released by an excess of free biotin [37]. In addition, in the study in question, Sano and Cantor reported that the mutant had weaker subunit interactions than that of wt streptavidin both in the presence and in the absence of biotin.

The residues in streptavidin which form the hydrogen bonds with biotin ureido oxygen were converted by Stayton's group to alanine (N23A, S27A and Y43A), phenylalanine (Y43F) or glutamate (N23E), and the affinities for biotin were 282, 114, 67, 6.9 and 69 times weaker than that of wt streptavidin [49]. In our similar approach with avidin, Tyr33 (equivalent to the Tyr43 of streptavidin) was mutated to alanine, phenylalanine, glutamine or histidine [50]. The results were in line with those reported for the analogous streptavidin mutants. The phenylalanine (Y33F) and histidine (Y33H) mutants displayed only a 4- and 6-fold decrease, respectively, in 2-iminobiotin affinity. The alanine (Y33A) mutant showed a 50-times weaker affinity toward 2-iminobiotin, whereas the affinity of the glutamine (Y33Q) mutant was too low to be measured reliably by the method used in the study. One important result was that histidine substitution changed biotin binding to being pH dependent. Interestingly, all the avidin mutants, except Y33F, also showed markedly reduced stability of the tetrameric quaternary structure in an SDS-PAGE-based stability assay as compared with that of wt avidin. The proteins were mainly monomeric even at relatively low temperatures. Biotin binding restored, however, the tetramers in assay conditions, but the stability of these biotin complexes was also impaired as compared with that of wt avidin.

In wt streptavidin, the Asp128 side chain carboxylate oxygen is hydrogen-bonded to one of the ureido nitrogen atoms of biotin. In addition, this oxygen is also involved in hydrogen bonds with side chain nitrogen atoms of Trp92 and Asn23. Furthermore, the other carboxylate oxygen is hydrogen-bonded to side chain nitrogen atoms of Trp108 and Gln24. Upon the conversion of D128A this network of interactions is broken [51]. The 3D structure of the mutant indicated that the hydrogen bond between Asn23 and the biotin ureido oxygen was lengthened to 3.8 Å, and that a water molecule had entered the binding site to replace the missing interactions [52]. It was also stated in a subsequent study by Stayton's group that the structure closely parallels a key intermediate observed in a simulated dissociation pathway of biotin from streptavidin [53].

The biotin-binding residues Ser45, Thr90 and Asp128 of streptavidin have been converted separately, in combinations of two, or of all three to alanines [54, 55]. These mutants showed a wide spectrum of affinities toward biotin ($K_d = 10^{-6}$–10^{-11} M). It was also reported that the mutants were partially or completely monomeric in an SDS-PAGE assay, and that the presence of biotin, at least partially, restored the tetramers of some mutants. In addition, the results indicated that modification of biotin-binding residues only could have a far-reaching effect on monomer-monomer interactions. However, in another independent study the S45A streptavidin mutant displayed an affinity toward biotin three orders of magnitude lower [56], but the quaternary 3D structure was almost unaltered as compared with that of wt streptavidin. This is somewhat contradictory to the results described above. The D128A mutant showed even more diverse results, since based on its 3D structure it was observed as a completely tetrameric form [51] and nonetheless, as a mainly monomeric form in another study [54]. These differences in results cannot be comprehensively explained, but the different production and purification methods (renaturation from inclusion bodies *versus* secreted) as well as different analytical approaches (calorimetry *versus* biosensor, X-ray crystallography *versus* SDS-PAGE assay) may explain some of them.

In a recent study Ting and coworkers [57] constructed a streptavidin that binds biotin in monovalent fashion by introducing three single point mutations to its biotin-binding site and mixing this mutant form with wt protein. The constructed N23A, S27D and S45A triple mutant (Table 1) showed a K_d of 1.2×10^{-3} M to free biotin but no binding to biotinylated cells. To create a monovalent protein the investigators fused a histidine tag to wt streptavidin, denatured the wt and mutant proteins in a 1 : 3 ratio following by the renaturation of the mixture. The renatured tetramers were captured by a nickel-nitrilotriacetic acid column and eluted by imidazole gradient. The biotin-binding affinity of the monovalent streptavidin was indistinguishable from the wt protein and was stable at 37 °C for 1 week. The monovalent streptavidin was used to label cell surface proteins with no cross-linking problem, such as was observed in the case when the wt tetravalent streptavidin was utilized.

The biotin-binding site of streptavidin has also been engineered by Sano and Cantor and their coworkers to alter the ligand specificity to favor 2-iminobiotin over biotin. This was accomplished by lowering the biotin affinity while retaining the 2-iminobiotin affinity virtually unaltered [58]. In the study in question two of the three amino acid residues, namely Asn23 and Ser27, which form hydrogen bonds with the ureido oxygen of biotin, were converted to alanine and aspartate or glutamate. The biotin affinity of the N23A and S27D mutants was reduced from that of wt streptavidin ($K_d = 2.5 \times 10^{-13}$ M) to a value around $K_d = 1 \times 10^{-4}$ M, whereas the 2-iminobiotin affinity was relatively well conserved and two orders of magnitude higher than that of biotin.

The biotin-binding site of streptavidin has moderate affinity toward a nine-amino-acid oligopeptide (Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly) called Strep-tag [59, 60],

Table 1. Mutagenesis studies made for avidin and streptavidin. The published mutations are shown. The affinity ratio shows the measured biotin dissociation constant for mutated protein divided by the value obtained for wt protein. The reported oligomeric state in solution is indicated as: M, monomeric; D, dimeric; T, tetrameric. T_m, reported transition melting temperature in differential scanning calorimetry analysis; T_r, reported transition temperature for the oligomeric disassembly in SDS-PAGE-based analysis; IR, immunological reactivity; ND, protein loses quaternary structure already at the room temperature.

Mutation	Affinity ratio vs wt	Oligo-mericity	T_m	T_r	Other properties (pI, PDB accession, etc.)	Refer-ences
Streptavidin						
wt	1 (K_d =4 × 10^{-14} M)	T	75.5	72	pI ≈ 6, PDB 1hqq, 1hxl, 1hxz, 1hy2, 1i9 h, 1lcv, 1lcw, 1lcz, 1luq, 1mk5, 1pts, 1r8t, 1rst, 1rsu, 1sld, 1sle, 1slf, 1slg, 1sre, 1srf, 1srg, 1srh, 1sri, 1srj, 1stp, 1str, 1sts, 1swa, 1swb, 1swc, 1swd, 1swe, 1vwa, 1vwb, 1vwc, 1vwd, 1vwe, 1vwf, 1vwg, 1vwh,, 1vwi, 1vwj, 1vwk, 1vwl, 1vwm, 1vwn, 1vwo, 1vwp, 1vwq, 1vwr, 2bc3, 2f01, 2gh7, 2iza, 2izb, 2izc, 2izd, 2ize, 2izf, 2izg, 2izh, 2izi, 21zj, 2izk, 2izl, 2rta, 2rtb, 2rtc, 2rtd, 2rte, 2rtf, 2rtg, 2rth, 2rti, 2rtj, 2rtk, 2rtl, 2rtm, 2rtn, 2rto, 2rtp, 2rtq, 2rtr	[7] [19, 135] For structural review, see [136]
E14S, Y22S, I30S, E51S, R53A, V55A, Y83G, E101S, N105S, E116S	5	T	–	ND	IR↓	[103]
W21antA[1]	↑↑[j]	–	–	–		[116]
W21atnDap	↑↑[j]	–	–	–		[118]
Y22antA	109	–	–	–		[116]
Y22atnDap	↑[k]	–	–	–		[118]
Y22dnsaF[s]	↑↑[j]	–	–	–		[117]
Y22S, E51S, Y83G	1	T	–	–	IR↓	[103]
Y22S, I30A, E51S, Y83G	5	T	–	–	IR↓	[103]
Y22S, E51S, R53A, Y83G, E116S	2	T	–	~40	IR↓	[103]
N23A	282[c]	T	–	–	PDB 1n4j, 1n43	[49]
N23A, S27D	–	T	–	–		[58]
N23A, S27D, S45A	3.0 × 10^{10}	T	–	–		[57]
N23E	69[c]	T	–	–	PDB 1n7y	[49]
L25antA	–[u]	–	–	–		[116]
L25atnDap	↑[k]	–	–	–		[118]
L25dnsaF	↑[k]	–	–	–		[117]
L25W, S45R	6.9 × 10^{4}[x]	–	–	–		[114]
L25W, S45W, L110W[v]	3.6 × 10^{5}[x]	–	–	–		[114]
L25W, S45Y, L110W[v]	5.4 × 10^{5}[x]	–	–	–		[114]
S27A	114[c]	T	–	–	PDB 1n9m, 1n9y	[49]
S27R, S45R	7.9 × 10^{5}[x]	–	–	–		[114]
S27R, S45R, L110W	3.3 × 10^{6}[x]	–	–	–		[114]
S27R, S45R, W120A	1.3 × 10^{6}[x]	–	–	–		[114]
Y43A	67[c]	T	–	–	PDB 1nc9, 1ndj, 1nbx	[49]
Y43antA	1.3 × 10^{5}	–	–	–		[116]
Y43atnDap	↑↑[j]	–	–	–		[118]
Y43dnsaF	–[u]	–	–	–		[117]
Y43F	7[c]	T	–	–	PDB 1swu	[49]
E44atnDap	↑↑[j]	–	–	–		[118]
E44dnsaF	↑↑[j]	–	–	–		[117]
E44I, S45G, V47R		T	–	>25	Strep-tagII	[26]
E44Q	4	T	–	–	IR↓	[103]
E44Q, E51Q, R53I, R84I	21	T	–	–	IR↓	[103]
E44V, S45T, V47R		T	–	>25	Strep-tagII, PDB 1kff, 1kl3, 1kl4, 1kl5	[26]
E44antA	↑[k]	–	–	–	Low yield	[116]
S45A	907[c]	T	78	–	PDB 1df8	[54, 56]

Table 1. (Continued).

Mutation	Affinity ratio vs wt	Oligo-mericity	T_m	T_r	Other properties (pI, PDB accession, etc.)	Refer-ences
S45A, T90A, D128A		M	–	–		[54]
S45W, L110W	4.2×10^4 [x]	–	–	–		[114]
S45Y, L110W	9.3×10^4 [x]	–	–	–		[114]
A46G, E51S, R53G	7	T	–	–	IR↓	[103]
V47G	–	–	–	–		[137]
N49antA	↑↑[j]	–	–	–	Low yield	[116]
N49atnDap	↑[k]	–	–	–		[118]
N49C	–	–	–	–	Allows conjugation	[109]
N49dnsaF	↑↑[j]	–	–	–		[117]
E51antA	↑[k]	–	–	–		[116]
E51atnDap	↑[k]	–	–	–		[118]
E51dnsaF	↑[k]	–	–	–		[117]
E51S	2	T	–	–		[103]
E51S, R53A, V55A, Y83G	10	T	–	–	IR↓	[103]
E51S, R53A, V55A, Y83G, E116S	1	T	–	–	IR↓	[103]
E51S, R53A, Y83G	1	T	–	–	IR↓	[103]
E51S, R53A, Y83G, E101, E116S	1	T	–	–	IR↓	[103]
E51S, R53A, Y83G, N105S, E116S	1	T	–	–	IR↓	[103]
E51S, R53A, Y83G, E116S	1	T	–	~40	IR↓	[103]
E51S, Y83G	1	–	–	~60	IR↓	[103]
E51S, Y83G, E101S	1	T	–	–	IR↓	[103]
S52antA	↑[k]	–	–	–		[116]
S52atnDap	–[u]	–	–	–		[118]
S52atnDap, 84ntrF[q]	2.5×10^5	–	–	–		[115]
S52dnsaF	↑↑[j]	–	–	–		[117]
Y54antA	1.8×10^4	–	–	–		[116]
Y54atnDap	↑↑[j]	–	–	–		[118]
Y54ntrF, 84atnDap[q]	2.5×10^5	–	–	–		[115]
V55R	–	(T)	–	> RT		[67]
V55T	–	T	–	> RT		[67]
V55T, T76R, L109T, V125R	4.7×10^6 [y]	M	–	ND		[67]
D61E		T	–	> RT		[102]
P64G	–	–	–	–		[137]
A65antA	↑↑[j]	–	–	–	Low yield	[116]
A65atnDap	↑↑[j]	–	–	–		[118]
A65dnsaF	↑↑[j]	–	–	–		[117]
A65R, T66G[i]	1[h]	T	–	ND		[96]
N69A, W120K	6.2×10^6 [y]	T	–	ND		[67]
W75dnsaF	–[u]	–	–	–		[117]
T76R	–	(M)	–	ND		[67]
T76R, V125R	4.3×10^6 [y]	M	–	ND		[67]
W79A	5.8×10^5	T	–	–		[36]
W79dnsaF	↑[k]	–	–	–		[117]
W79F	180/3[d]	T	–	–	PDB 1swh, 1swj, 1swk	[36]
K80atnDap	↑↑[j]	–	–	–		[118]
K80dnsaF	↑↑[j]	–	–	–		[117]
K80G	–	T	–	–		[137]
N81P	–	–	–		Not functional	[93]

Table 1. (Continued).

Mutation	Affinity ratio vs wt	Oligo-mericity	T_m	T_r	Other properties (pI, PDB accession, etc.)	Refer-ences
Y83atnDap	↑[k]	–	–	–		[118]
Y83dnsaF	↑↑[j]	–	–	–		[117]
Y83G	1	T	–	–	IR↓	[103]
R84atnDap	–[u]	–	–	–		[118]
R84dnsaF	↑↑[j]	–	–	–		[117]
T90A	–	–	–	–		[54]
T90I	↑↑[bb]	–	–	–		[57]
W92dnsaF	↑↑[j]	–	–	–		[117]
Y96A	–	–	–	–	SP[v]	[111]
Y96S	–	–	–	–		[111]
Y96D	–	–	–	–		[111]
V97A	–	–	–	–	MP[v]	[111]
E101A	–	–	–	–		[111]
E101antA	↑↑[j]	–	–	–		[116]
E101atnDap	↑[k]	–	–	–		[118]
E101dnsaF	↑↑[j]	–	–	–		[117]
E101Q, E116Q, K121 M, K132M	1	T	–	–		[103]
R103A	–	–	–	–	SP[v]	[111]
R103G	–	–	–	–		[111]
R103P	–	–	–	–		[111]
I104A	–	–	–	–	SP[v]	[111]
I104V	–	–	–	–		[111]
I104T	–	–	–	–		[111]
N105A	–	–	–	–	MP[v]	[111]
N105D	–	–	–	–		[111]
N105T	–	–	–	–		[111]
T106A	–	–	–	–	LP[v]	[111]
Q107A	–	–	–	–	Enriched[v]	[111]
Q107E	–	–	–	–		[111]
Q107P	–	–	–	–		[111]
W108A	–	T	–	–	SP[v]	[36, 111]
W108antA	↑↑[j]	–	–	–		[116]
W108atnDap	↑↑[j]	–	–	–		[118]
W108dnsaF	↑↑[j]	–	–	–		[117]
W108F	209/2[d]	T	–	–	PDB 1swl, 1swn,	[36]
W108G	–	–	–	–		[111]
W108S	–	–	–	–		[111]
L109A	–	–	–	–	LP[v]	[111]
L109V	–	–	–	–		[111]
L109P	–	–	–	–		[111]
L110A	–	–	–	–	SP[v]	[111]
L110V	–	–	–	–		[111]
L110P	–	–	–	–		[111]
T111A	–	–	–	–	LP[v]	[111]
S112A	–	–	–	–	MP[v]	[94, 111]
S112C	–	–	–	–		[94]
S112D	–	–	–	–		[94]
S112E	–	–	–	–		[94]
S112F	–	–	–	–		[94]
S112G	–	–	–	–		[94, 137]

Table 1. (Continued).

Mutation	Affinity ratio vs wt	Oligo-mericity	T_m	T_r	Other properties (pI, PDB accession, etc.)	Refer-ences
S112H	–	–	–	–		[94]
S112I	–	–	–	–		[94]
S112K	–	–	–	–		[94]
S112L	–	–	–	–		[94]
L112M	–	–	–	–		[94]
S112N	–	–	–	–		[94]
S112P	–	–	–	–		[94]
S112Q	–	–	–	–		[94]
S112R	–	–	–	–		[94]
S112T	–	–	–	–		[94]
S112V	–	–	–	–		[94]
S112W	–	–	–	–		[94]
S112Y	–	–	–	–		[94]
T114A	–	–	–	–	LP[v]	[111]
T114antA	–[u]	–	–	–	Low yield	[116]
T114atnDap	↑↑[j]	–	–	–		[118]
T115A					Enriched[v]	[111]
E116A					LP[v]	[111]
E116C	↑[z]	–	–	–	Allows conjugation	[105]
A117antA	–[u]	–	–	–	Low yield	[116]
A117atnDap	↑[k]	–	–	–		[118]
N118A	–	–	–	–	LP[v]	[111]
N118D	–	–	–	–		[111]
N118T	–	–	–	–		[111]
W120A	2.9×10^6, 8.6×10^4[x]	T	–	–	LP[v], PDB 1swq, 1swr	[36, 111, 114]
W120antA	532	–	–	–		[116]
W120atnDap	–[u]	–	–	–		[118]
W120dnsaF	–[u]	–	–	–		[117]
W120D(OMc)[o]	↑↑[p]	–	–	–		[116]
W120E(OMc)[n]	1.0×10^4	–	–	–		[116]
W120F	223/78[d]	T	–	–	PDB 1swo, 1swp	[36]
W120G	–	–	–	–		[111]
W120K	1.5×10^6[e]	D	–	ND	PDB 1nqm	[15]
W120mchA[m]	4.2×10^3[e]	–	–	–		[116]
W120S	–	–	–	–		[111]
K121A	–	–	–	–	LP[v]	[111]
K121T	–	–	–	–		[111]
K121E	–	–	–	–		[111]
S122A	–	–	–	–	MP[v]	[111]
S122G	–	–	–	–		[91]
T123A	–	–	–	–	LP[v]	[111]
L124A	–	–	–	–	MP[v]	[111]
L124antA	–[u]	–	–	–	Low yield	[116]
L124atnDap	↑↑[j]	–	–	–		[118]
L124dnsaF	↑↑[j]	–	–	–		[117]
L124P	–	–	–	–		[111]
L124R	–	–	–	–	PDB 1rxh	[80]
L124V	–	–	–	–		[111]
V125A	–	–	–	–	MP[v]	[111]
V125R	–	–	–	ND		[67]

Table 1. (Continued).

Mutation	Affinity ratio vs wt	Oligo-mericity	T_m	T_r	Other properties (pI, PDB accession, etc.)	Refer-ences
V125T	–	(T)	–	>RT		[67]
H127A	–	–	–	–	MP[v]	[111]
H127C	–	T	–	–		[68]
H127D	–	T	–	–		[68, 111]
H127P	–	–	–	–		[111]
D128A	1032[c]	–	–	–	MP[v], PDB 1swt, 1sws	[51, 54, 111]
D128dnsaF	↑↑[j]	–	–	–		[117]
T129A	–	–	–	–	MP[v]	[111]
F130A	–	–	–	–	SP[v]	[111]
F130S	–	–	–	–		[111]
F130V	–	–	–	–		[111]
T131A	–	–	–	–	MP[v]	[111]
K132A	–	–	–	–	MP[v]	[111]
K132E	–	–	–	–		[111]
K132T	–	–	–	–		[111]
V133A	–	–	–	–	MP[v]	[111]
S139C	–	–	–	–	Allows conjugation	[106]
Miscellaneous streptavidins						
CP51/46	5.7×10^6	T	–	–	PDB 1swf, 1swg	[72]
SCD ($M5L_L$, $S45L_L$, $W120K_L$)[aa]	–	–	–	–	Selected by panning w/biotin matrix	[77]
SCD ($F29L_L$, $W120K_L$, $K134E_L$, $A138T_L$, $G68S_L$, $K80E_R$, $G113S_R$) (C2)	3.3×10^8	pD[g]	–	–	K_d (B4F) = 1.6×10^{-10} M	[77]
SCD C2 tetramer	8.3×10^7	pT[g]	–	–	K_d (B4F) = 8.0×10^{-11} M	[77]
SCD ($F29L_L$, $W120K_L$, $S136T_L$, $G142E_L$, $G48D_R$, $S52T_R$, $S62N_R$, $R103K_R$) (E2)	3.8×10^7	pD[g]	–	–	K_d (B4F) = 3.0×10^{-11} M	[77]
SCD E2 tetramer	3.2×10^8	pT[g]	–	–	K_d (B4F) = 8.0×10^{-11} M	[77]
SCD ($S45L_L$, $W120K_L$,)	–	–	–	–	Selected by panning w/biotin matrix	[77]
SCD ($W120K_L$)	1.7×10^8	pT[g]	–	–	K_d (B4F) = 1.2×10^{-10} M	[77]
SCD ($W120K_L$, $V31L_R$, $S122P_R$)	–	–	–	–	Selected by panning w/biotin matrix	[77]
M2 (chimeric SA-AVD)	–	T	–	–	PDB 1rxj	[80]
M3 (chimeric SA-AVD+L124R)	–	T	–	–	PDB 1rxk	[80]
Avidin						
WT	1 ($K_d = 6 \times 10^{-16}$ M)	T	85.5	58	p*I* 10.5, PDB 1avd, 1ave, 1ij8, 1ldo, 1ldq, 1lel, 1rav, 1vyo, 2avi, 2a5b, 2a5c, 2a8g	[6, 7]
R2A, K3E, K9E	1	T	–	ND	p*I* 7.9	[38]
R2A, K3E, K9E, R122A, R124A	16	T	–	ND	p*I* 5.9	[38]
R2A, K3E, K9E, R26N, R59A, R122A, R124A	2	T	–	65	p*I* 4.7	[38]
R2A, K3E, K9E, N17I, R26N, R59A, R122A, R124A	1	T	–	60	pI 4.7, NG	[104]
K3E, K9E, R26D, R124L	1[dd]	T	–	–	PDB 2cam	[39]
K3E, K9D, R122A, R124A	–	T	–	–	p*I* 5.4	[38]
K3E, K9D, R122A, R124A	23	T	–	ND	p*I* 7.2	[38]
C4A, C83Y	8	T	76.4	–		[69]

Table 1. (Continued).

Mutation	Affinity ratio vs wt	Oligo-mericity	T_m	T_r	Other properties (pI, PDB accession, etc.)	Refer-ences
S16A	13[b]	T	84.3	55		[75]
N17I	2	T	–	60	NG	[104]
R26N, R59A	2	T	–	60	p*I* 9.0	[38]
Y33A	50	T	–	ND		[50]
Y33F	4	T	–	60		[50]
Y33H	6	T	73.0	ND		[50]
Y33Q	–	T	–	ND		[50]
T35A	238[b]	T	82.6	55		[75]
N54A	1	T	–	40		[64]
N54A, M96A, V115A, I117A	–	M/T	–	ND		[64]
N54A, N69A, M96A, V115A, I117A	–	M/T	–	ND		[64]
D86C, I106C	8	T	74.4	–		[69]
D86C, I106C, I117C	12	T	94.7	–		[69]
M96A, V115A, I117A	4	T	–	40		[64]
M96H	–	M/T	–	55		[70]
V115H	–	M/T	–	ND		[70]
W110K	4.5×10^7[e]	D	–	ND	PDB 1nqn	[15]
W110K, N54A	1.3×10^8[f]	M	–	ND		[65]
K111I	1.5[cc]	T	76.5	ND		[121]
I117C	3	T	98.6	–		[69]
I117H	–	T	97.0[a]	65		[70]
I117Y	3	T	97.5	–		[129]
R122A, R124A	7	T	–	60	p*I* 9.4	[38]
Miscellaneous avidins						
ChiAVD	5	T	96.5	–		[129]
ChiAVD, I117Y	3	T	111.1	–		[129]
cpAvd5 → 4	1	T	72.7	45		[73]
cpAvd6 → 5	3	T	65.6	40		[73]
dcAvd	2	pT[g]	80.2	40	PDB 2c4i	[73]
dcAvd($I117C_{5\rightarrow4}$)	0.6[ee]	pT[g]	87.7	60		[75]
dcAvd(I117C5 → $4S16A_{6\rightarrow5}$)	12[ff]	pT[g]	87.7	60		[75]
dcAvd($I117C_{5\rightarrow4}Y33H_{6\rightarrow5}$)	52[ff]	pT[g]	81.6	ND		[75]
dcAvd($I117C_{5\rightarrow4}T35A_{6\rightarrow5}$)	34[ff]	pT[g]	89.6	60		[75]
scAvd	1[ee]	pT[g]	83.1	–		[76]

[a] Nordlund, Hytönen, Nyholm & Kulomaa, unpublished; [b] Obtained by combining data from calorimetric analyses; [c] From analysis employing his-tagged wt streptavidin; [d] Ratio of 2-iminobiotin affinity compared with wt at pH 8 and pH 10; [e] Ratio of K_d measured for surface immobilized biotin vs value reported by Green; [f] K_d of mutant determined using intrinsic fluorescence quenching; [g] Pseudotetramer (pT) or pseudodimer (pD) in solution; [h] Ratio of measured dissociation rate constants; [i] Includes two amino acids adjacent to mutagenized sites; [j] Significant decrease in biotin-binding affinity based on dot-blot analysis; [k] Decrease in biotin-binding affinity based on dot-blot analysis; [l] Original residue substituted with nonnatural amino acid L-2-anthrylananine (antA); [m] Original residue substituted with nonnatural amino acid γ-(7-methoxycoumarin-4-yl)homoalanine (mchA); [n] Original residue substituted with nonnatural amino acid γ-(7-methoxycoumarin-4-yl)methyl L-glutamate [E(OMc)]; [o] Original residue substituted with nonnatural amino acid β-(7-methoxycoumarin-4-yl)methyl L-aspartate [D(OMc)]; [p] Affinity decreased more than 2000-fold; [q] Original residues substituted with nonnatural amino acids β-anthraniloyl-L-α,β-diaminopropionic acid (atnDap) and *p*-nitrophenylalanine (ntrF); [s] Original residue substituted with nonnatural amino acid β-2,6-dansyl-aminophenylalanine (dnsaF); [t] Original residue substituted with nonnatural amino acid β-anthraniloyl-L-α,β-diaminopropionic acid (atnDap); [u] No significant decrease in biotin-binding affinity based on dot-blot analysis; [v] LP, low preference (1–3-fold) of wt residue over alanine in this position in shotgun mutagenesis; MP, moderate preference (>3-fold); SP, strong preference (>10-fold); enriched; alanine residue enriched over wt residue in shotgun scanning selection procedure; [x] Determined by measuring the binding of monobiotinylated Fab to surface-immobilized streptavidin. $K_d = 2.5 \times 10^{-13}$ M was used for wt to calculate the ratio; [y] Determined by measuring the binding of streptavidin to a surface-immobilized biotinylated BSA. $K_d = 2.5 \times 10^{-13}$ M was used for wt to calculate the ratio; [z] The dissociation of biotin was faster at 37 °C compared with wt; [aa] The mutations in the subunits L and R are shown according the numbering of wt streptavidin; [bb] >50% dissociation from a biotin-fluorescein conjugate in 1 h at 37 °C (wt streptavidin dissociated less than 10% in 12 h at 37 °C); [cc] Difference in biotin dissociation rate constant at 50 °C; [dd] No significant difference in competition assay; [ee] Dissociation rate of fluorescent biotin conjugate vs wt; [ff] Dissociation rate of fluorescent biotin conjugate vs wt estimated for mutated subunit.

which was obtained from a pool of random oligopeptides displayed as C-terminal fusions on a V_H domain of a F_V fragment of an antibody. This tag has been used as an affinity tag to purify proteins produced as Strep-tag fusions in different expression systems. Strep-tag is readily released from streptavidin by the addition of excess biotin or biotin analogues, and only the histidine and glutamine residues occupy part of the space taken up by biotin in the binding site. In addition, a conformational change was observed in the 3D structure of the streptavidin strep-tag complex [61], involving opening of the loop between β-strands three and four. Strep-tag suffered from the restriction of being fully functional only as a C-terminal fusion. The sequence was therefore further modified and optimized, and a terminus-independent peptide, Strep-tagII (Asn-Trp-Ser-His-Pro-Gln-Phe-Glu-Lys), was obtained. This tag, however, displayed lower affinity toward streptavidin than the original Strep-tag, and therefore streptavidin itself was subjected to modification to increase the affinity. For this purpose the residues 44-47 (Glu-Ser-Ala-Val) in the loop between β-strands three and four were subjected to random mutagenesis [26]. Two of the screened streptavidin mutants showed an improved affinity of more than one order of magnitude ($K_d \approx 1 \times 10^{-6}$ M) toward Strep-tagII. The mutant bearing the Val-Thr-Ala-Arg stretch as residues 44-47 also showed enhanced performance in the purification of fusion proteins containing Strep-tagII when coupled to a chromatographic support.

Interface mutants

The contact patterns between the (strept)avidin subunits are structurally fairly well defined (Fig. 4). Attempts have therefore been made to produce modified monomeric and dimeric forms by breaking certain interactions in the tetramers, and in some cases even in adjustable fashion. On the other hand, the stability of (strept)avidin quaternary structure has in some cases been improved by stabilizing and/or optimizing their interface contacts.

On the basis of sequence information obtained from the fibropellin avidin-like domain, the functional one-to-two interface residues Trp110 in avidin and Trp120 in streptavidin have been converted to lysines [15]. The affinity of the resultant avidin (W110K) and streptavidin (W120K) mutants toward biotin dropped by several orders of magnitude ($K_d \approx 1 \times 10^{-8}$ M). The nature of the binding was rapidly reversible, since an excess of free biotin released most of the bound mutant from the biotin surface within minutes. In addition, both mutants were shown to exhibit stable dimeric quaternary structures in solution. The 3D structures of these dimeric (strept)avidins have recently been resolved [62, 63]. Interestingly, they appeared in the crystals to be tetramers almost identical to the wt proteins, probably indicating a concentration-dependent dimer-tetramer transition.

The one-to-three (Fig. 4c, d) and one-to-four (Fig. 4e, f) monomer-monomer interactions of avidin have been weakened by mutating first the one-to-three interface residues (M96, V115 and I117), and then the two key-residues (N54 and N69) of the one-to-four interface to alanines [64]. A series of four mutants was described, all of which showed reduced quaternary structure stability, particularly in the absence of biotin. Two of the mutants bearing the one-to-three alanine substitutions with N54A or with both N54A and N69A were completely monomeric, even at room temperature in an SDS-PAGE assay, and also in gel filtration FPLC in the absence of biotin. However, the presence of biotin induced tetramerization of the mutants, rendering them almost as stable as wt avidin tetramers.

In a subsequent study, a fully monomeric avidin form, monoavidin, was produced by mutation of two interface residues: Trp110 in the one-to-two interface was mutated to lysine, and Asn54 in the one-to-four interface was converted to alanine [65]. The affinity for biotin binding of the mutant decreased from the K_d of approximately 10^{-15} M of the wt tetramer to the K_d of approximately 10^{-7} M of monoavidin. In addition, conversion of the tetramer to a monomer caused increased sensitivity to proteinase K digestion. The antigenic properties were also changed such that monoavidin was recognized only partially by a polyclonal antibody, and two different monoclonal antibodies [66] failed entirely to recognize the avidin monomer. Monoavidin binds biotin in rapidly reversible manner, and may therefore be useful for applications both *in vitro* and *in vivo*.

Qureshi and Wong were able to turn streptavidin into a soluble monomer by converting two biotin-binding residues, Thr90 and Asp128, simultaneously into alanines [55]. They reported the K_d of the resultant mutant to be 1.3×10^{-8} M toward biotin and showed successful utilization of the immobilized mutant in the purification of biotinylated cytochrome C from a bacterial extract, and then its mild elution by desthiobiotin. In a more recent study, the same group used a rational approach to change several amino acid residues in all subunit interfaces to generate electrostatic repulsion and steric hindrance between subunits [67]. Mutations of Val55, Thr76 and Val125 residues into either arginine or threonine were utilized. The effect of the single substitutions in weakening the subunit interactions followed the order V55T < V55R = V125T < V125R < T76R. A double mutant (T76R, V125R) was adequate for the complete monomerization of streptavidin. To render monomeric streptavidin more hydrophilic, a quadruple mutant (T76R, V125R, V55T and L109T) was constructed. These monomeric streptavidins, based on interface residue modifications, showed approximately four times weaker affinity toward biotin when compared with monomeric streptavidin, where monomerization was achieved by mutating biotin-binding residues T90A

and D128A [54, 55, 67]. In the same study Wu and Wong [67] also constructed a streptavidin mutant where they mimicked our strategy to monomerize avidin. A double mutant (Q95A, W120K) (analogous to the monoavidin containing mutations N54A and W110K) was, however, oligomeric but showed a strong biotin-binding affinity collapse, similar to our W120K streptavidin mutant [65]. This underlines the fact that, although in many respects avidin and streptavidin show characteristics quite similar to each other, direct analogy for achieving desired mutants is not always possible between these two proteins.

His127 of streptavidin, which faces His127 from the neighboring subunit (in the one-to-three interface, Fig. 4d), has been converted to aspartate to create electrostatic repulsion between subunits one and three [44]. This was intended to prevent the formation of the wt tetramer and result in a dimeric form of the protein. However, the mutant in fact formed non-functional aggregates. To reduce the hydrophobicity of the protein (*i.e.* to allow a soluble dimeric structure) it was further modified by deleting part of the loop between β-strands seven and eight containing the amino acid residues Gly113–Trp120. Owing to this, a β-turn (Ser112–Lys121) connecting β-strands seven and eight was assumed to form. This combined H127D and loop deletion mutant was shown to be dimeric, but it refolded only in the presence of excess biotin and disassembled into non-functional monomers in the absence of the ligand. The biodistribution of this dimeric streptavidin has been examined in a subsequent study and its clearance from the circulation was observed to be faster than that of wt core streptavidin, and its accumulation in the liver and kidney was also diminished [45].

To enhance the streptavidin tetramer stability, the His127 of streptavidin has been converted to cysteine, lysine and aspartate. The cysteine mutant was shown to form intermonomeric disulfide bridges [68]. Furthermore, introduced cysteine residues were successfully used to chemically link the subunits utilizing 1,3-dibromoacetone. The modified forms evidenced enhanced stability in both cases according to an SDS-PAGE assay after heat treatment and a biotin-binding assay in guanidine hydrochloride. The lysine mutant was designed to form hybrid tetrameric streptavidins with the aspartate mutant, in which the lysine ε-amino group from one subunit would form a favorable electrostatic interaction with the β-carboxyl group of aspartate from the neighboring subunit [68]. In addition, the lysine and aspartate side chains were chemically cross-linked using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) and the mutant was reported to be more stable than wt streptavidin.

Analogously, we have shown that tetrameric chicken avidin can be stabilized by introducing intermonomeric disulfide bridges between its subunits in one-to-three and one-to-four interfaces [69]. These covalent bonds had no major effects on the biotin-binding properties of the respective mutants. Moreover, one of the mutants, Avd-ccci (D86C, I106C and I117C), maintained its tetrameric integrity even in denaturing conditions. The avidin forms Avd-ci (I117C) and Avd-ccci, which have native-to-denatured transition midpoint temperatures (T_m) of 98.6 °C and 94.7 °C, respectively, in the absence of biotin, are likely to find use in applications where extreme stability or minimal leakage of subunits is required. Furthermore, we showed that the intramonomeric disulfide bridges found in wt avidin affect its stability. The mutant Avd-nc (C4A, C83Y), where this covalent bonding was removed, had in the absence of biotin a lower T_m (76.4 °C) than wt avidin (85.5 °C). In the presence of biotin, however, it showed stability similar (T_m = 118.2 °C) to wt avidin (T_m = 118.5 °C).

To turn the subunit association and biotin binding of avidin into pH-sensitive phenomena, three amino acid residues (Met96, Val115 and Ile117) have been individually replaced in avidin with histidines in the one-to-three interface, and in combination with a histidine conversion in the one-to-two interface (W110H) [70]. The rationale behind these kinds of mutations is that the pK_a value of histidine residues in a polypeptide chain is at pH between 6 and 7, meaning that at higher pH values histidine is deprotonated. The single point mutant I117H was interestingly even more thermostable (at pH 7.0) than wt avidin, showing a T_m of 97 °C and 124.2 °C in the absence and presence of biotin, respectively (Hytönen et al., unpublished). The single replacements M96H and V115H in the one-to-three interface had a clear effect on the quaternary structure of avidin, since the subunit associations of these mutants became pH dependent. The histidine replacement in the one-to-two interface affected the biotin-binding properties of the mutants, in particular the reversibility of binding, and protein-ligand complex formation of the double mutants was pH sensitive. Concerning applications, the most interesting mutants were Avm(M96H, W110H), Avm(M96H) and Avm(I117H, W110H), whose behaviors were consistently predictable. In the case of Avm(M96H, W110H), biotin was able to induce formation of tetramers at pH 7.2 and 11, but a lowering of pH to 4 caused its dissociation into monomers regardless of the presence of biotin. This should allow easy detachment of bound biotinylated materials from immobilized Avm(M96H, W110H) simply by lowering the pH and adding free biotin. Avm(M96H) was a tetramer both in the absence and in the presence of ligand at pH 7.2. This mutant was a monomer at pH 4 in the absence of biotin, the addition of which, however, induced its tetramerization at this pH. It is thus possible to regulate its quaternary structure assembly alternatively by changing pH in the absence of biotin, or by adding biotin at a low pH. The mutant Avm(I117H, W110H) exhibited quaternary structure characteristics similar to wt avidin, being a stable tetramer at all pH values studied. Consequently, its rapid

biotin-binding reversibility at low pH might be a valuable property in some applications.

Topology modifications

A common approach to the study of protein folding and the significance of secondary structure topology is the creation of circularly permuted forms of the proteins in question (for a review, see [71]). In this approach, the original N and C termini are usually brought together with a linker peptide, after which the new termini are introduced, typically into a loop region. In most cases proteins withstand these modifications fairly well, exhibiting no radical alterations in their structure or function. These kinds of circular permutation studies have also been conducted with avidin and streptavidin.

The loop residues between β-strands three and four of streptavidin were investigated by Stayton and coworkers [72] by creating a circularly permuted form where the original termini were joined by a tetrapeptide linker. The β-strand four preceding Glu51 was then used as the new N terminus and β-strand three following Ala46 was converted to the new C terminus (Fig. 6). The objective was to determine the importance of the flexible loop between β-strands three and four, which undergoes an open-to-closed conformational change upon biotin binding. As a result the backbone topology was changed, and residues 47-50, including Asn49, which in wt streptavidin forms a hydrogen bond between main-chain amide nitrogen and the biotin valeryl moiety carboxylate [4, 16], were deleted. Despite the modification, this circularly permuted streptavidin exhibited well-conserved tertiary and quaternary structures, but a severely reduced affinity toward biotin (K_d approximately 10^{-7} M) was observed, although the position of biotin in the binding pocket remained almost unaltered in X-ray structure [72].

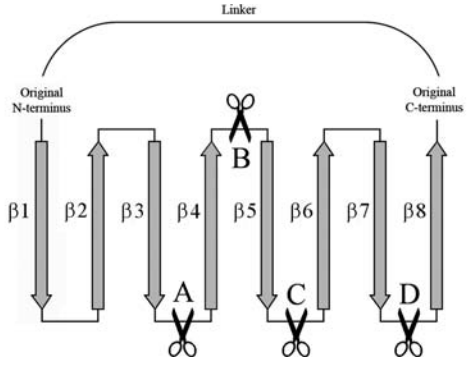

Figure 6. Topologically modified avidin forms. Four different circularly permuted avidin or streptavidin forms have been described. They have either been produced as free subunits, or different circularly permuted domains have been joined in one polypeptide chain. In each case the original N and C termini were joined by a linker and the new termini were introduced into the loop regions highlighted in the figure by a pair of scissors A–D. Introduction of the new termini to position A produces (strept)avidin beginning with β-strand four and ending after β-strand three [72]. Similarly, in the B case the avidin mutant begins with β-strand five and ends after β-strand four [73, 75, 77]. Moreover, in C the sequence begins with β-strand six and ends after β-strand five [73, 75]. Finally, in D the streptavidin form begins with β-strand eight and ends after β-strand seven [77]. The biotin-binding site of avidin resides at the same end of the antiparallel β-barrel as the new termini in cases A, C and D, reflecting the biotin-binding affinity of the resultant proteins.

In another study, the present authors designed two distinct circularly permuted forms of avidin with the aim of constructing a fusion avidin containing two biotin-binding sites in one polypeptide [73]. The old N and C termini of wt avidin were connected to each other via a short glycine/serine-rich linker, and the new termini were introduced into two different loops (Fig. 6). This enabled the creation of the desired fusion construct using a short linker peptide between the two different circularly permuted subunits. The circularly permuted avidins (circularly permuted avidin 5 → 4 or cpAvd5 → 4, and circularly permuted avidin 6 → 5 or cpAvd6 → 5), and their fusion, pseudotetrameric dual chain avidin or dcAvd (EMBL: AJ616762), were biologically active, *i.e.* they showed biotin binding and also displayed structural characteristics similar to those of wt avidin [73]. The better-conserved biotin-binding affinity of these circularly permuted avidin forms, as compared with the above-described circularly permuted streptavidin, was evident. It is most probably due to the fact that in cpAvd5 → 4 the new termini were introduced in the opposite end of the biotin-binding site of the barrel, and in cpAvd6 → 5 at the biotin-binding end of the barrel, and no residues were deleted. The binding residues were therefore retained in the circularly permuted avidins, which was not the case or the aim in the streptavidin variant produced by Chu et al. [72]. However, the latter circularly permuted avidin, cpAvd6 → 5, showed slightly reduced binding affinity, which may be caused by the increased freedom of the biotin-binding residues close to the termini to move. Another potential explanation is the unique conformation of Phe72 in cpAvd6 → 5 compared with other avidin forms [74].

Dual chain avidin (dcAvd) was modified further to contain two distinct independent ligand-binding sites or domains within a single polypeptide chain. The dcAvd scaffold was then used to generate further engineered avidins where the neighboring biotin-binding sites exhibit different affinities for biotin [75]. To lower the affinity in the half of the binding sites, mutations S16A, Y33H and T35A (Table 1) were subjected to one of the binding domains of dcAvd. The pseudotetramer, *i.e.* a dimer of the modified dcAvds, was found to have two high (wt-like) and two lower (modified) affinity biotin-binding sites. These dual-affinity dcAvd molecules should provide totally new possibilities in avidin-biotin technology, where they may have uses as novel bioseparation tools, carrier proteins or nanoscale adapters.

The next rational step, single-chain avidin (scAvd), having four biotin binding domains, was constructed by fusion of two dcAvds [76]. ScAvd showed biotin-binding and thermal stability properties similar to wt avidin. The scAvd-encoding DNA construct (EMBL: AJ966780) contains four circularly permuted avidin domains, plus short linkers connecting the four domains into a single polypeptide chain. In contrast to wt avidin, which contains four identical avidin monomers, this enables each of the four avidin domains to be modified independently by rational and random protein engineering. Therefore, scAvd scaffold can be used to construct spatially and stoichiometrically defined pseudotetrameric avidin molecules showing different domain characteristics. In addition, scAvd could be used as a fusion partner, since it provides a unique non-oligomeric structure that is fully functional with four high-affinity biotin-binding sites.

Streptavidin has also been modified to have two binding sites in one polypeptide chain (EMBL: AY884152; [77]). In this case two different circularly permuted forms of streptavidin (Fig. 6), one beginning with β-strand eight and ending after β-strand seven, was fused to another form beginning with β-strand five and ending after β-strand four. The novel streptavidin form was also further modified by introducing point mutations, and the mutants were expressed employing phage display and panning with biotinylated beads (EMBL: AY884153, EMBL: AY884154; [77]). Surprisingly, it was observed that these novel streptavidin forms displayed high affinity towards biotin-4-fluorescein conjugate (K_d = 10^{-10}–10^{-11} M), whereas the affinity to biotin was significantly reduced in these proteins (K_d = 10^{-5}–10^{-6} M) compared with wt streptavidin. Furthermore, these proteins showed both dimeric (pseudotetrameric) and monomeric (pseudodimeric) appearance in fast-protein liquid chromatography analysis, and the oligomerization process was found to be biotin dependent in the case of monomeric forms, such that they appeared as dimers in the presence of biotin.

Chimeric forms of (strept)avidin

Chicken avidin has been found to possess pseudocatalytic activity, being capable of enhancing the hydrolysis of biotinyl *p*-nitrophenyl ester (BNP), *i.e.* hydrolyze an ester bond between the biotin and nitrophenyl groups [78]. In contrast, bacterial streptavidin prevents the hydrolysis reaction in high alkaline conditions, where some spontaneous hydrolysis is observed. To establish whether this property could be moved from avidin to streptavidin, a chimeric form of streptavidin has recently been created in a study in which certain structural features of avidin were transferred to streptavidin by Livnah and his coworkers [79]. In this chimera, the loop between β-strands three and four in streptavidin (residues 48–52, GNAES) was replaced with the corresponding loop from avidin (residues 38–45, TATSNEIK) in the mutant called M1. Furthermore, a point mutation L124R was introduced to the M2 streptavidin sequence (analogous to Arg114 in avidin), as this was thought to be important for the catalysis based on the previous structural analyses of the complexes of (strept)avidin and biotin or biotin conjugate [78]. This arginine in avidin presumably repels the BNP substrate into a conformation that favors its hydrogen-bond interaction with Lys111 from the adjacent monomer, which is equivalent to Lys121 in streptavidin. Finally, the third mutant M3 was a combination of the mutants M1 and M2. The 3D structure of these chimeric streptavidin–avidin forms was determined by X-ray crystallography [80]. The M1 mutant showed significantly improved hydrolytic activity, but only above a pH value of 10.5. The M2 mutant was already markedly active at pH 8 and above, similarly to avidin. However, combination of the avidin-derived loop and point mutation L124R was required to yield fully avidin-like activity [79]. In a more detailed study, analysis was made of the molecular and structural determinants contributing to the pseudocatalytic reaction. It was found that in another member of the avidin protein family, AVR4, different amino acid residues can perform the same function and that subtle differences in the active-site structure can result in alternative modes of reaction [81].

In addition to avidin, the chicken avidin gene family also includes seven other members known as the avidin-related genes (*AVR1–AVR7*) [82–86]. One of the products of these genes, AVR4, has been found to be the most heat-stable biotin-binding protein thus far characterized (T_m = 106.4 °C) [87]. To transfer the high thermostability of AVR4 to avidin, a chimeric avidin protein, ChiAVD, containing a 21-amino acid segment of AVR4 including the region corresponding to L3,4, β-strand four and L4,5, was constructed (roughly between scissors A and B in Fig. 6). This chimeric avidin-AVR4 was found to be significantly more stable (T_m = 96.5 °C) than native avidin (T_m = 83.5 °C), and its biotin-binding properties resembled those of AVR4. Furthermore, an AVR4-inspired point mutation, I117Y, was introduced to the interface between subunits one and three in avidin (Fig. 4). This mutation alone significantly increased the thermostability of the avidin mutant (T_m = 97.5 °C) without compromising its high biotin-binding properties. Combination of these two modifications yielded a hyperthermostable protein form (T_m = 111.1 °C), which was named ChiAVD(I117Y).

Towards the use of (strept)avidin as an enzyme

In chemistry, two stereoisomers are said to be enantiomers if they are mirror images of each other. In respect of biochemical activity, synthesis of pure stereoisomeric compounds is important. For this enantioselective cataly-

sis is utilized as an efficient tool (for an introduction, see [88]). Artificial metalloenzymes combine an organometallic structure with a protein [89]. The structure of the protein thus partially determines the environment of the catalytic reaction. (Strept)avidin has also been used as a structural scaffold for this purpose. In the first attempt, chicken avidin was used to catalyze the asymmetric hydrogenation of α-acetamidoacrylic acid to *N*-acetylalanine. A catalytic moiety, achiral diphosphinerhodium(I), was attached to the protein *via* biotin [90]. This system yielded a significant (~40%) excess of the S enantiomer. Streptavidin has since been tested for the same purpose and it worked efficiently, yielding a 94% excess of the R enantiomer [91]. It has been shown that the efficiency of the catalytic moiety is increased in the presence of (strept)avidin [91]. To establish whether the high isoelectric point of avidin is the reason for lower catalytic efficiency, an avidin form carrying mutations K3E, K9D, R122A, R124A was produced in *Pichia pastoris* [32] and used in experiments. This genetic neutralization of avidin, however, showed no significant change in its properties as a scaffold in enantioselective catalysis [91].

The structure of the catalytic moiety used has been varied to broaden the methodology [92]. To obtain more efficient tools for these purposes, (strept)avidin has also been modified. Streptavidin mutants V47G, P64G, K80G and S112G were produced in *E. coli* [93]. Their activity as scaffolds in enantioselective hydrogenation in combination with different types of biotinylated catalytic moieties was then tested [91]. Mutations close to the active site were found to have more effect on the selectivity. For example, in the case of a certain biotinylated catalytic moiety (biotin-4^{ortho}-2), streptavidin mutant V47G favored production of the R enantiomer (44%), whereas wt protein favored production of the S enantiomer (28%). In a recent study, streptavidin residue Ser122 was changed to all 19 other residues [94]. The resulting 20 proteins were combined with the biotinylated catalyst precursors to obtain 360 different artificial metalloenzymes. These were used to measure their efficiency in catalyzing hydrogenation of α-acetamidoacrylic acid and α-acetamidocinnamic acid. In the study in question, the most interesting differences in substrate selectivity in terms of catalytic efficiency were observed with biotin–3^1–2 in combination with aromatic residues in position 112. For example, mutant S112Y catalyzed production of the S enantiomer, whereas wt streptavidin catalyzed production of the R enantiomer when biotin–3^3–1 was used as a chemical moiety.

Other modifications

The β-barrel structure of streptavidin has been used as a scaffold to display cell-adhesive hexapeptide sequences derived from osteopontin and fibronectin, which contain the RGD cell adhesion domain [95, 96]. In the initial construct, the ATD sequence of streptavidin (amino acids 65–67) in the loop between β-strands four and five was converted to RGD by site-directed mutagenesis. This construct did not, however, promote cell adhesion in an *in vitro* experiment with rat aortic endothelial and human melanoma cells, possibly due to inadequate exposure or a sterically unfavorable structure of the domain. For this reason, the RGD flanking regions present in osteopontin and fibronectin were also introduced into the constructs, and the final sequences promoting integrin-dependent cell adhesion were GRGDSP and GRGDSV, respectively. Wt streptavidin contains an RYDS sequence stretch (amino acids 59–62) homologous to the fibronectin RGDS. In the above-described study [96] wt streptavidin did not show adhesion to the melanoma and endothelial cells. In other studies, however, Chinese hamster ovary cells, M4 murine melanoma cells, ADP-activated platelets and $CD4^+$ lymphocytes have shown integrin-mediated cell adhesion, possibly *via* this homologous stretch [97–99]. Furthermore, the kidney takes up a large proportion of streptavidin administered *in vivo*, which is a disadvantageous property in several medical applications [100, 101]. It has been assumed that the kidney uptake *in vivo* is mainly integrin-mediated and dependent on the wt RYDS domain. This conception is supported by results obtained in a study in which the RYDS was mutated to RYES and the resultant point-mutated D61E streptavidin showed markedly reduced cell attachment *in vitro* [102].

Reduced antibody response to streptavidin mutants has been described in a site-directed mutagenesis study [103]. The objective there was to create a less antigenic but otherwise structurally and functionally normal form of streptavidin, which could be administered repeatedly *in vivo*. Meyer and colleagues [103] hypothesized that the physical forces that stabilize antigen-antibody interactions might also be important in antigen recognition by the immune system. They therefore assumed that if the epitope residues were suitably replaced, the resulting protein would be less immunoreactive and also bear less antigenicity. They constructed a series of 37 mutants in which a collection of surface-exposed aromatic, large hydrophobic and charged residues were mainly substituted with serine, glycine or alanine residues. The remaining conversions were more or less conservative, such as glutamic acid to glutamine, aspartic acid to asparagine and lysine to methionine. One of the mutants (E51S, R53A, Y83G, E116S) showed less than 10% immunoreactivity compared with that of wt streptavidin, but its antigenicity was not reduced and it elicited a strong immune response in rabbits. Another mutant (E14S, Y22S, I30S, E51S, R53A, V55A, Y83G, E101S, N105S, E116S) was described to be only 20% as antigenic as streptavidin. In addition, it showed a loss of cross-reactivity and rabbits

immunized with it or streptavidin failed to recognize the alternative antigen.

To expand the usefulness of avidin in applications where its electrostatic, nonspecific binding is detrimental, a series of charge-reduced avidin mutants, with p*I*s ranging from 9.4 to 4.7, has been described [38]. The series contains six different mutants, which have two to six amino acid substitutions from positive to neutral or negative. The mutations were designed using the evolutionary approach, and the R2A and K3E substitutions were designed on the basis of analogous residues present in streptavidin, according to a sequence alignment. Furthermore, the K9E, R26N and R59A substitutions were designed using the sequence information of the avidin-related proteins in a similar manner, whereas the R122A and R124A substitutions were designed on the basis of a careful inspection of the 3D structure [5]. The mutation K9D (and not the designed K9E) present in the p*I* 7.2 mutant originated in a PCR error. All the mutants displayed an affinity toward 2-iminobiotin almost as high as that of wt avidin, indicating that the substituted surface residues had neither major functional nor far-reaching structural effects on the biotin-binding site. In addition to this, all mutants displayed considerably stable quaternary structures particularly in the presence of biotin, which suggested that the monomer-monomer interactions also remained quite intact despite the multiple modifications. However, some mutants showed lowered stability in the presence of SDS, but the most acidic mutant bearing all the mutations was even more stable (in the presence of SDS) than wt avidin. These findings were supported by an independent study in which a similar acidic (p*I* 5.5) avidin, with four substitutions (K3E, K9E, R26D and R124L) was crystallized and the 3D structure revealed that the inversion of the exposed electrostatic charges had no significant effect on the overall structure [39]. Moreover, the p*I* 4.7 avidin mutant [38] has been further modified by an N17I substitution. Due to this AVR-derived mutation the *N*-glycosylation recognition site of avidin was abolished and the resultant non-glycosylated mutant showed excellent nonspecific binding properties [104].

To render streptavidin-biotin interaction more adjustable and rapidly reversible, Stayton and coworkers [105, 106] conjugated stimuli-responsive polymers to streptavidin. The polymers used respond to environmental stimuli such as changes in temperature, pH and light, by changing their conformation and physical state. To conjugate these polymers in a precise site in streptavidin the investigators mutated the desired amino acids into cysteines. In one of these mutants, Glu116, which allows conjugation close to the biotin-binding site, was converted to cysteine residue, whereas the S139C mutation led the conjugation to occur more distantly from the binding pocket. The biotin-binding switching was highly efficient in the case of the E116C mutant but less successful in the case of S139C. Applications for this kind of smart molecular switches can be found in the fields of separation and diagnostic methods as well as targeted drug and gene delivery [107]. A high off-rate mutant, S45A, was also used in this context to release reversibly biotinylated macromolecules [108].

Mutation N49C has been introduced to streptavidin to allow conjugation of the protein with imaging agent [109]. This residue is located in the close vicinity of the carboxylic tail of streptavidin-bound biotin (Fig. 1).

A combinatorial alanine scanning method called shotgun scanning [110] has also been used to analyze the functional contribution of the 38 C-terminal residues of streptavidin [111]. In this method, the amino acid side chains studied were preferentially allowed to vary only between the wt and alanine. The phage-displayed mutant streptavidin pool was subjected to biotin-binding selection and the biologically active forms were selected and sequenced. For each amino acid position studied, the wt/alanine ratio was determined. When a large ratio was observed, *i.e.* the wt side chain was markedly preferred over the alanine substitution in functional mutants, the side chain was assumed to have a central role in the biological activity. The phage-display construct was designed to produce tetrameric streptavidin, and therefore both streptavidin subunits and streptavidin phage coat protein P8 fusions were expressed from the same construct. This was accomplished using an amber (the stop codon UAG) suppressor *E. coli* strain and an amber stop codon between streptavidin and the phage coat protein P8 coding regions [112]. The shotgun scanning results for streptavidin were largely in line with previous results obtained from single-point site-directed mutagenesis studies on biotin contact residues. Additionally, new residues with long-distance impact for biotin binding or stability of the tertiary and quaternary structures were resolved. For example, mutations Y96A, R103A, I104A and F130A had a high impact for the function of streptavidin [111].

To enhance the performance of streptavidin-based solid-phase assays, a hexapeptide containing a single cysteine residue has been fused to the C terminus of streptavidin [113]. The readily reactive sulfhydryl group of the cysteine was used to immobilize the streptavidin variant on maleimide-coated solid surfaces. The hexapeptide sequence contained a short four-residue linker (Gly-Gly-Ser-Gly) followed by the cysteine residue and at the C terminus a proline that was assumed to protect the protein from exopeptidases. According to the design only two specific, cysteine-mediated, covalent bonds between the mutant and a solid surface were assumed to form. This could leave at least two binding sites per tetramer fully accessible to biotinylated macromolecules, which would be a major improvement when compared with the capacity obtained with wt streptavidin immobilized covalently *via* its amino groups. Furthermore, immobilized wt streptavidin

shows quite different biotin-binding characteristics when compared with those of streptavidin in solution [113]. The higher binding capacity of the streptavidin variant was confirmed, and in addition, it showed only slightly different biotin-binding characteristics when compared with those of streptavidin in solution [113].

How to inactivate (strept)avidin?

Since (strept)avidin has high stability and the biotin-binding property comprises several additive factors, the protein allows one to use fairly robust modifications without significantly affecting its function. However, some reported modifications have yielded more or less inactive (strept)avidin forms, which give insight into structurally important features of these proteins.

An approach in which the biotin-binding activity of streptavidin was rationally decreased showed that substitutions Xaa → Trp or Xaa → Arg in the biotin-binding site were not able to totally block the biotin binding [114]. For example, the most inactive streptavidin, containing mutations S27R, S45R and L110W, still showed affinity towards the biotin surface in the micromolar range (Table 1). This is notable considering the extremely good fit between the ligand-binding site and biotin (Fig. 3).

An interesting approach in expanding the chemical and biological diversity of proteins is position-specific incorporation of non-natural amino acids carrying a variety of special side-groups. Aminoacylation of tRNA with non-natural amino acids has been achieved by directed evolution of aminoacyl-tRNA synthetases or ribozymes. Codons have been extended to include four-base or five-base codons or non-natural base pairs [115]. There would seem to exist significant differences in the biotin-binding capacity of resultant mutants when non-natural amino acids are incorporated into streptavidin. For example, substitution in positions 85 and 87 has always yielded inactive streptavidin in respect of biotin binding [116–118]. In contrast, some positions appear to be quite suitable targets for this kind of substitution, for example residues 25 and 51, which have yielded high-affinity biotin-binding streptavidins after substitutions with various types of non-natural amino acids. Incorporation of non-natural amino acids in streptavidin have yielded virtually inactive mutants in the sense of biotin binding based on dot-blot analysis in the case of W75antA, W79antA, K80antA, Y83antA, R84antA, N85antA, H87antA, W92antA [116], W21dnsaF, N85dnsaF, H87dnsaF, T114dnsaF, A117dnsaF [117] and W75atnDap, W79atnDap, N85atnDap, H87atnDap, W92atnDap [118]. Similarly, a double mutant (52atnDap, Y83ntrF) was found to be inactive [115]. The abbreviations used for the non-natural amino acids are as follows: antA, 2-anthrylalanine; dnsaF, 2,6-dansyl-aminophenylalanine; atnDap, β-anthraniloyl-L-α,β-diaminopropionic acid; ntrF, *p*-nitrophenylalanine.

Comparison of non-functional streptavidin forms has shown that mutations D67S, V97A, R103S and V133A are most probably responsible for the failures in the protein-folding process [103]. Visual inspection of 3D structure of streptavidin shows that residue Asp67 is exposed to solvent in L4,5 and forms a hydrogen bond with the main chain oxygen of residue Ser69. The reason why this mutation causes problems in the folding process is difficult to specify. Similarly, Val97 and Arg103 are located in L6,7 and exposed to solvent. Of those, Val97 does not seem to form significant interactions with other residues in the sense of protein folding, whereas Arg103 forms hydrogen bonds with residues of the side chain O of Thr131 and of the main chain O of Ala102. Notably, Avrantinis and coworkers [111] found that the Val97 and Arg103 residues are particularly selected against alanine in streptavidin shotgun mutagenesis. Val133 is located in the C terminus of streptavidin and packs against the Tyr22 side chain ring.

In this view, Table 1 could be used as a guide when this kind of mutagenesis work is planned for (strept)avidin. Some of the residues, although located on the surface of the proteins, are sensitive to substitutions such that the mutated protein loses the biological activity to a considerable extent. Examples of such residues are Ala65 residing in loop L4,5 and Leu124, which is located in the neighborhood of residue Val125, which is an important residue forming the one-to-three subunit interface (Fig. 4).

General rules for estimation of results of amino acid substitution in (strept)avidin are:

The aromatic amino acid residues forming the hydrophobic core and the biotin-binding cavity of the protein are sensitive to substitutions and the substitution often results in inactive protein in respect of biotin binding or a significant decrease in the binding affinity (residues Trp21, Trp75, Trp79, Trp92, Trp108 and Trp120 in streptavidin).

Some specific (charged or polar) residues in the protein interfaces seem to be important for the activity (fold) of the protein (His87, Asn85, Lys80, Arg84 in streptavidin).

The single hydrogen bonds are not critical for biotin-binding affinity (SaS27A, SaY43F, AvdY33F, AvdS16A, AvdT35A, SaS45A).

The marked similarity of functionally important residues in avidin and streptavidin makes it possible to extend the understanding obtained mainly from streptavidin studies to avidin and other biotin-binding proteins.

Trp110/120 provides an interesting target in the production of novel (strept)avidin forms. Although this residue is known to be particularly important for the high biotin-binding affinity of these proteins (Table 1), it seems to be a good target for incorporation of exotic substituents

[116, 118]. Furthermore, these novel Trp → X proteins appear to evince interesting features giving them potential in the development of new tools for life science.

In the case of non-natural amino acids, the high divergence between the original and the new residue may result in loss in activity, although the position appears to resist substitution with other types of residue (as in the case of Ser52 located in the protein surface, loop L3,4; Thr114 located in L7,8). If non-natural amino acids are incorporated, the use of a chemically mimicking structure may result in a more functional protein. For example, mutation W92dnsaF yielded a somewhat active protein, whereas mutants W92antA and W92atnDap were virtually inactive as regards biotin binding. Analogously W79dnsaF evinced biotin-binding activity, whereas mutants W79atnDap and W79antA showed no significant biotin-binding activity. Careful examination of the 3D structure combined with the existing data could thus provide good indications for the selection of residues to be incorporated. T114 and L124 are interesting from another point of view, since it seems that these positions yield different activity of the final product depending on the residue type incorporated, the smaller residues being more active. Examination of the 3D structure of streptavidin reveals that these residues are located in the protein surface but have only a limited amount of free space around them, and therefore the incorporation of large residues in these positions could result in physical hindrance and defects in the protein folding.

Conclusions – views for future research

Research on biotin-binding proteins hitherto has been strongly focused on chicken avidin and bacterial streptavidin. The biochemical properties of these proteins have been determined using various techniques, and a large number of modified forms of them have been described. However, relatively few of these products have, at least so far, found their uses in commercial applications. One example of a promising new product in the (strept)avidin-biotin product family is StrepTactin, which is a modified streptavidin showing high affinity toward a polypeptide ligand [119]. One probable reason for the poor success hitherto to find further practical applications for (strept)avidin technology is the lack of a cost effective expression and purification system for these modified proteins. The development of many efficient protocols for the production of (strept)avidin during the past few years may lead to an increase in availability of commercialized products in the future [26, 32, 33].

In addition to avidin and streptavidin, alternative biotin-binding proteins have been found and characterized. These include the avidin-related proteins AVR1-AVR7 from the chicken [87, 120, 121], chicken egg yolk biotin-binding proteins [122–125], avidins from other birds [126, 127] and novel biotin-binding proteins, such as bradavidin [128], from bacteria other than genus *Streptomyces.* These proteins have two potential impacts for the development of improved (strept)avidins. Firstly, they may as such have beneficial properties that render them superior to avidin or streptavidin in specific applications. Secondly, they can serve as a source of ideas when developing advanced (strept)avidin forms. The development of a non-glycosylated, acidic avidin mutant [104] and avidin-AVR chimeras [129] are examples of the latter.

As described in previous sections, modification of (strept)avidin has until recently been limited to the homotetrameric approach. This limits the feasibility of the methods, since different types of (strept)avidin subunits can be obtained in the same target only by mixing the tetrameric proteins, thus limiting the quality of the final product. More importantly, the number of distinct types of molecules in the target (for example the surface of a particle) is not a simple result of concentration of components, this because the binding behavior, solubility and other properties can be affected by modification of the protein. In this sense, dcAvd [73] and scAvd [76] offer powerful scaffolds to further develop (strept)avidin technology for applications demanding improved and new fully controlled protein molecules. The dual-affinity avidins described offer a proof of principle of this concept [75].

Biotinylation is an extremely important biological event that is used to tune the central biochemical functions in the cells. Biotin is known to be linked to different types of carboxylases, decarboxylases and transcarboxylases, and also other biotinylation targets have been found, for example histones [130]. Biotinylation is performed by a specific enzyme, biotin protein ligase BPL, and it occurs in certain polypeptide sequences [131, 132]. Recent studies have shown that it is possible to use fairly short sequences, which are efficiently biotinylated *in vivo* [132]. These biotinylation sequence 'tags' should offer good potential in future biochemical studies. However, the determinants of the specificity of BPL-mediated biotinylation are only partially known [132]. One interesting finding was recently made by Choi-Rhee and coworkers [133], who observed less specific biotinylation occurring due to a mutation in the *E. coli* biotin holoenzyme synthetase/bio receptor BirA. A mutagenesis of BirA leading to a new sequence specificity could thus be obtained, offering an efficient tool for life science. For example, a shorter biotinylation signal should offer better feasibility for the biotinylation of target protein *in vivo*. Furthermore, since biotinylation signals are present in cells naturally, the artificial mutated BPL/BirA biotinylating a novel sequence could offer a more useful means of producing biotinylated proteins not disturbing the normal biological machinery of the cell. Recently BirA has proved capable

of attaching the ketone form of biotin to the biotinylation signal in proteins [134].

The knowledge gained hitherto from studies concerning (strept)avidin could also be used to develop biotinylation systems. The availability of biotin-detection tools and the variety of modified avidins together with the development of biotinylation systems offers a tool kit for future studies in life science.

What have we learnt from the modification and engineering of (strept)avidin? These versatile proteins serve as a basic tool in various methods. Their robustness and their high affinity to biotin make their use easy and simple. Via modification there are ever increasing possibilities of application, and novel forms can be fairly easily generated since there are efficient expression methods available. The latest success in the field of (strept)avidin engineering would indicate that the enormous potential of this system has not yet been fully utilized. A simple protein-ligand pair can be modified for employment in methods ranging from medicine to biology and from chemistry to physics, providing a scaffold for novel functions. It seems that engineered (strept)avidins are opening the way to 'next-generation' protein tools. This review summarizes the present knowledge, and hopefully also gives new insights for further successful research.

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