Engineering of chicken avidin: a progressive series of reduced charge mutants

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Abstract Avidin, a positively charged egg-white glycoprotein, is a widely used tool in biotechnological applications because of its ability to bind biotin strongly. The high p*I* of avidin (~10.5), however, is a hindrance in certain applications due to non-specific (charge-related) binding. Here we report a construction of a series of avidin charge mutants with p*I*s ranging from 9.4 to 4.7. Rational design of the avidin mutants was based on known crystallographic data together with comparative sequence alignment of avidin, streptavidin and a set of avidin-related genes which occur in the chicken genome. All charge mutants retained the ability to bind biotin tightly according to optical biosensor interaction analysis. In most cases, their thermal stability characteristics were indistinguishable from those of the wild-type avidin. Our results demonstrate that the charge properties of avidin can be modified without disturbing the crucial biotin-binding activity.

Key words: Avidin; Protein engineering; Charge mutant; Avidin-biotin technology

1. Introduction

Avidin is a basically charged, tetrameric glycoprotein found in the chicken egg white. Throughout the years, avidin has become a frequently used tool in numerous biotechnological applications, including different localization, diagnostic and separation technologies [1]. Recently avidin has also found its use in affinity-based targeting of drugs and imaging agents with promising results [2–5]. All these applications are generally based on the high affinity (K_d ~10^{-15} M) [6] avidin has for biotin, a low-molecular-weight vitamin, which can be readily attached to biologically active binders and detectable probes. This strong interaction with biotin, combined with the exceptional stability and four biotin-binding sites of avidin (one per subunit) has created the inherent utility and the versatility of the avidin-biotin technology.

Avidin is, however, a positively charged glycoprotein (p*I* ~10.5) [6], which possesses eight arginine and nine lysine residues [7]. The high p*I* of avidin and the presence of carbohydrate residues have been a constant hindrance to its use in some applications, due to non-specific binding (mostly charge-related) to extraneous material. For this reason streptavidin, a non-glycosylated and neutrally charged bacterial counterpart of avidin [8], has become the preferred choice in such applications. The preference of streptavidin over egg-white avidin has prevailed, despite the fact that avidin is more hydrophilic, contains more lysine residues for potential attachment of probes, and is considerably more abundant and cheaper than streptavidin.

In the present work, we wanted to investigate whether the p*I* of avidin can be reduced using protein engineering, without disturbing significantly the biotin-binding activity or the stability characteristics of avidin. We used sequence comparison of streptavidin [9] and recently cloned avidin-related (*avr*) genes [10], together with the crystallographic structure of avidin [11,12] to design the changes. This approach has allowed us to generate a series of fully functional avidin mutants with p*I*s ranging from 9.4 down to 4.7. These reduced charge mutants bind biotin in a manner similar to that of wild-type avidin and also display clearly reduced non-specific binding characteristics. Therefore, this study also offers new possibilities for the applications of avidin-biotin technology. Preliminary results of this work were presented at the 8th European Congress on Biotechnology, Budapest 17–21, Hungary, August 1997, Abstracts, p. 158.

2. Materials and methods

2.1. Site-directed mutagenesis and construction of recombinant baculoviruses

Mutagenesis of avidin cDNA [13] was accomplished by the PCR-based megaprimer method [14] using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). The oligonucleotides for mutagenesis were purchased from either KEBO Lab (Espoo, Finland) or from MedProbe (Oslo, Norway). After digestion with *Bgl*II and *Hin*dIII (Promega, Madison, WI, USA), the PCR fragments were subcloned into the *Bam*HI/*Hin*dIII digested pFastBAC1 donor vector (Gibco-BRL, Gaithersburg, MD, USA). The mutations were confirmed by double-stranded sequencing using Sanger's dideoxynucleotide chain termination procedure with an automated DNA sequencer (ALF, Pharmacia Biotech). The recombinant baculoviruses were generated using the Bac-To-Bac baculovirus expression system according to the manufacturer's instructions (Gibco-BRL). The primary virus stocks were amplified for large-scale production of avidin mutants and the titers of the stocks were determined by a plaque assay procedure [15].

2.2. Expression and purification of avidin mutants

Insect cells, Sf9 (ATCC CRL 1711), were maintained as a suspension culture in serum-free Sf-900 II SFM medium (Gibco-BRL) and infected with recombinant baculoviruses at m.o.i. of 0.5–2 pfu/cell. The infection was allowed to proceed for 24 h, after which the culture medium was collected by centrifugation (100×*g*, 22°C, 5 min) and replaced with fresh biotin-free medium. After the medium change, the infection was continued for another three days. The purification of each avidin mutant was carried out by affinity chromatography using 2-iminobiotin-agarose as previously described by Airenne et al. [16].

2.3. Protein analysis

Electrophoretic analysis was carried out using 15% (w/v) SDS-

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PAGE with discontinuous buffer system [17]. After electrophoresis, proteins were either stained with Coomassie brilliant blue or blotted onto nitrocellulose membrane for immunostaining according to Airenne et al. [16]. Isoelectric focusing was performed using polyacrylamide gels with pH gradient ranging from 3 to 10. An aliquot of avidin mutants (5 μg) together with the p*I* standards (Bio-Rad) was applied to the gel, and following the run the proteins were visualized by Coomassie staining. The quaternary status of avidin was analyzed by FPLC on a Superose 12 column (Pharmacia) using an LKB HPLC system. A sample (20 μg in 100 μl of phosphate buffer with 0.65 M NaCl, pH 7.2) was applied, and chromatography was carried out at a flow rate of 0.5 ml/min, using the same ionic strength in the equilibration and running buffers. The column was calibrated using bovine γ-globulin, BSA, avidin standard, ovalbumin, ribonuclease and cytochrome *c* as molecular weight markers.

2.4. Interaction analysis of avidin mutants

Binding kinetics were measured using optical biosensor technology (IASyS Manual+, Affinity Sensors, Cambridge, UK). The measurements (R_{eq}, in arc seconds, a measure of the mass on the surface) were carried out using either a commercial biotin cuvette (Affinity Sensors) or by immobilizing 2-iminobiotin onto the carboxymethyldextran cuvette using *N*-hydroxysuccinimide activation. 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 NaCl at room temperature. The iminobiotin cuvettes were regenerated with 20 mM HCl. The measurements using the biotin cuvette were carried out using PBS with 1 M NaCl as a binding buffer at room temperature. The kinetic rate constants for association (k_{on}) and dissociation (k_{off}) or the dissociation constant (K_d) were calculated using the Fast Fit program package (Affinity Sensors).

2.5. Thermal stability of avidin mutants

Purified avidin mutants, in the presence or absence of an excess of biotin, were combined with sample buffer (0.125 M Tris-HCl, pH 6.8/4% (v/v) SDS/20% (v/v) glycerol/0.004% (v/v) bromophenol blue/10% (v/v) 2-mercaptoethanol) and incubated at selected temperatures, before being subjected to SDS-PAGE as described by Bayer et al. [18]. The gels were stained using Coomassie brilliant blue. The stability of the proteins was followed by dissociation of the tetramer to the monomeric form.

2.6. Non-specific binding assay

Successive dilutions (1 μg, 200 ng, 40 ng and 8 ng) of a DNA sample (salmon-sperm DNA or pGEM plasmid DNA) were applied to nitrocellulose strips. The DNA was fixed to the strips by UV irradiation, after which the strips were quenched using 5× Denhardt's solution. The sample (20 μg in 1 ml PBS) was then added to a strip and incubated at room temperature for 90 min. After that, the strips were washed with PBS/0.05% Tween 20 solution and stained immunochemically as described by Airenne et al. [16].

3. Results

3.1. Design of avidin mutants

In order to reduce the positive charge of avidin, a series of six mutants was constructed with p*I* range from 9.9 down to 4.7, as calculated theoretically from amino acid sequences using the GCG program package (Genetics Computer Group, Madison, WI, USA). The alterations were done by changing selected basic amino acids to neutral or acidic ones using site-directed mutagenesis (Table 1) and the proteins were named according to the actual p*I*. The selection of amino acids for lowering the p*I* was based on sequence comparison of avidin, streptavidin and avidin-related proteins (AVR), combined with the available crystallographic data of avidin. All the altered amino acids are surface residues and, according to structural information, have no major role in biotin binding or stability of the avidin tetramer. Where possible, we tried to change arginine residues rather than lysines, due to the importance of their terminal amino groups for derivatization of avidin in many applications.

3.2. Expression and purification of avidin mutants

We have shown previously [16] that avidin can be produced efficiently in Sf9 insect cells, using a baculovirus expression system. The expression of the avidin mutants was generally comparable to that of the wild type, although some correlation between production and the number of the mutations was evident – AvdpI9.4 and AvdpI7.9 being best in this respect (data not shown). All the mutants were purified to 95% purity (judged from SDS-PAGE gel) in one step, using affinity chromatography with 2-iminobiotin as a capturing ligand. For successful purification it was essential to use biotin-free culture medium; if biotin is present in the medium, it will block the binding sites of avidin/mutants thus hampering subsequent affinity purification and possible use of the avidin derivative in applied systems.

3.3. Protein chemical analysis

SDS-PAGE analysis showed that the native avidin and charge mutants separated into three components (data not shown), which presumably represented different stages of post-translational modification of glycosylated avidin [16]. There were minor changes in the migration pattern of certain mutants (especially AvdpI4.7), probably due to the extensive charge differences. The isoelectric points of the avidin mutants were determined with isoelectric focusing, using a pH gradient from 3 to 10 (Fig. 1). The experimental results corresponded well with the values theoretically calculated from the amino acid sequences (Table 1). The differences in the case of AvdpI9.4 and AvdpI9.0 are probably due to poor resolution in the upper part of the pH gradient. The experimental value for avidin could not be determined with this system, since its p*I* is over 10, which is out of range of the gel system used in this study. The quaternary status of the charge mutants, in the presence and in the absence of biotin, was examined by FPLC on a Superose 12 column (data not shown). Comparison of the elution profiles with molecular mass standards

Table 1
The physiochemical properties of the avidin charge mutants

Name	Mutations	p*I* calculated	p*I* experimental	K_d (M)[a]
Avd	None	10.4	n.d.	2.0×10^{-8}
AvdpI9.4	R122A, R124A	9.9	9.4	1.4×10^{-7}
AvdpI9.0	R26N, R59A	9.3	9.0	3.1×10^{-8}
AvdpI7.9	R2A, K3E, K9E	8.1	7.9	2.3×10^{-8}
AvdpI7.2	K3E, K9D, R122A, R124A	7.2	7.2	4.5×10^{-7}
AvdpI5.9	R2A, K3E, K9E, R122A, R124A	5.9	5.9	3.1×10^{-7}
AvdpI4.7	R2A, K3E, K9E, R26N, R59A R122A, R124A	4.7	4.7	3.5×10^{-8}

[a]Dissociation constant for 2-iminobiotin at pH 9.5; n.d., not determined.

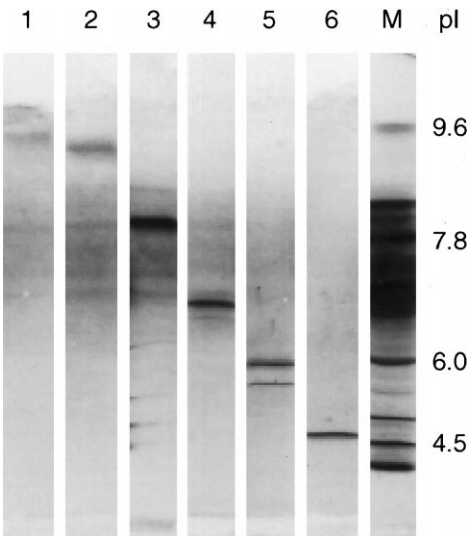

Fig. 1. Isoelectric focusing of avidin charge mutants. Samples (5 μg) of different charge mutants were applied to polyacrylamide gel with pH gradient from 3 to 10. Following the run, proteins were visualized by Coomassie brilliant blue staining. Lanes: 1: AvdpI9.4; 2: AvdpI9.0; 3: AvdpI7.9; 4: AvdpI7.2; 5: AvdpI5.9; 6: AvdpI4.7; M: p*I* standard.

showed that all the mutants behaved similarly to native avidin (56.6 kDa) forming stable tetramers (the molecular masses of different mutants varied from 55.3 to 60.5 kDa).

3.4. Biotin-binding characteristics of avidin mutants

All six charge mutants bound strongly to 2-iminobiotin, since they were efficiently affinity purified in a single step using this moiety as a capturing ligand. To further characterize the biotin-binding affinities of different mutants, an optical biosensor instrument (IASyS Manual+) was used. When attempts were made to measure the binding constants of mutant proteins to immobilized biotin, no dissociation was observed (data not shown). This indicated that all the mutant proteins exhibited very high affinity constants for biotin – similar to the tenacious binding by the wild-type protein. To obtain precise information concerning the possible differences in affinities among the various mutants, 2-iminobiotin was used as a ligand instead of biotin. Avidin binds 2-iminobiotin at elevated pH with a lower, readily measurable level of affinity. The analysis of the binding curves of the wild-type avidin and different reduced charge mutants, each in several concentrations, were used to determine their dissociation constants to 2-iminobiotin at pH 9.5 (Fig. 2). The calculated K_d = 2.0×10^{-8} M for avidin is in good agreement with that reported previously by Green [6]. The K_d values for different mutants varied from 1.4×10^{-7} M to 3.5×10^{-8} M (Table 1), suggesting that their biotin-binding properties are quite similar to that of wild-type avidin.

3.5. Thermal stability of avidin mutants

In order to determine whether the mutations affected the thermal stability of the avidin tetramer, the avidin mutants and native avidin were diluted in SDS-containing buffer and heated to temperatures between 25°C and 100°C in the absence and presence of biotin. It has been shown previously that under the conditions of this assay biotin stabilizes the quaternary structure of avidin [18]. The transition temperatures for biotin-saturated mutants should therefore be higher than those of the biotin-free derivatives. Biotin-free native avidin starts to dissociate into monomers at temperatures over 57°C (Fig. 3A), whereas with biotin the transition begins only at temperatures near 100°C (Fig. 3B). The results of the thermostability experiment showed that of all the mutant avidins AvdpI4.7 (Fig. 3C and D) was the most stable. Surprisingly, this mutant appeared to be slightly more stable than wild-type avidin. AvdpI9.4 and AvdpI9.0 showed dissociation profiles similar to that of native avidin, but biotin-free AvdpI7.9, AvdpI7.2 and AvdpI5.9 appeared to dissociate, presumably into monomers, at room temperatures when SDS was present (data not shown). Interestingly, in the presence of biotin they behaved in a manner similar to that of native avidin, requiring temperatures near 100°C to dissociate.

3.6. Non-specific binding to DNA

A dot-blot assay was used to investigate the status of the charge mutations with respect to the non-specific binding properties of avidin (data not shown). Wild-type avidin bound strongly to both single- and double-stranded DNA. Most of this binding seems to be charge-related, since there was a correlation between lowering the isoelectric point and reduced binding to DNA; AvdpI4.7 showed the lowest levels of binding to DNA.

4. Discussion

During the last two decades, avidin has evolved into a key component in many biotechnological applications such as affinity-based separations and diagnostic assays [1]. Applications of avidin-biotin technology utilize the extraordinary affinity avidin has for biotin. While the usefulness of avidin is impressive, there are some drawbacks associated with its utilization in some applications. Most of these problems are related to the high p*I* of avidin. For example the alkaline nature of avidin can cause charge-related binding to DNA and cell surfaces, which may hinder its use in certain circum-

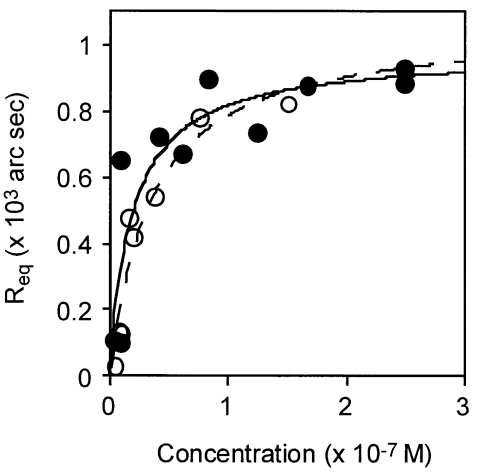

Fig. 2. Interaction of native avidin (closed circles) and AvpI4.7 (open circles) with 2-iminobiotin. Various concentrations of avidin or mutant were added to 2-iminobiotin-coated cuvettes, and binding was measured in pH 9.5 buffer at room temperature using an IASyS optical biosensor. The equilibrium response (R_{eq}) is plotted vs. protein concentration. The K_d of the protein is equal to the concentration at $R_{max}/2$.

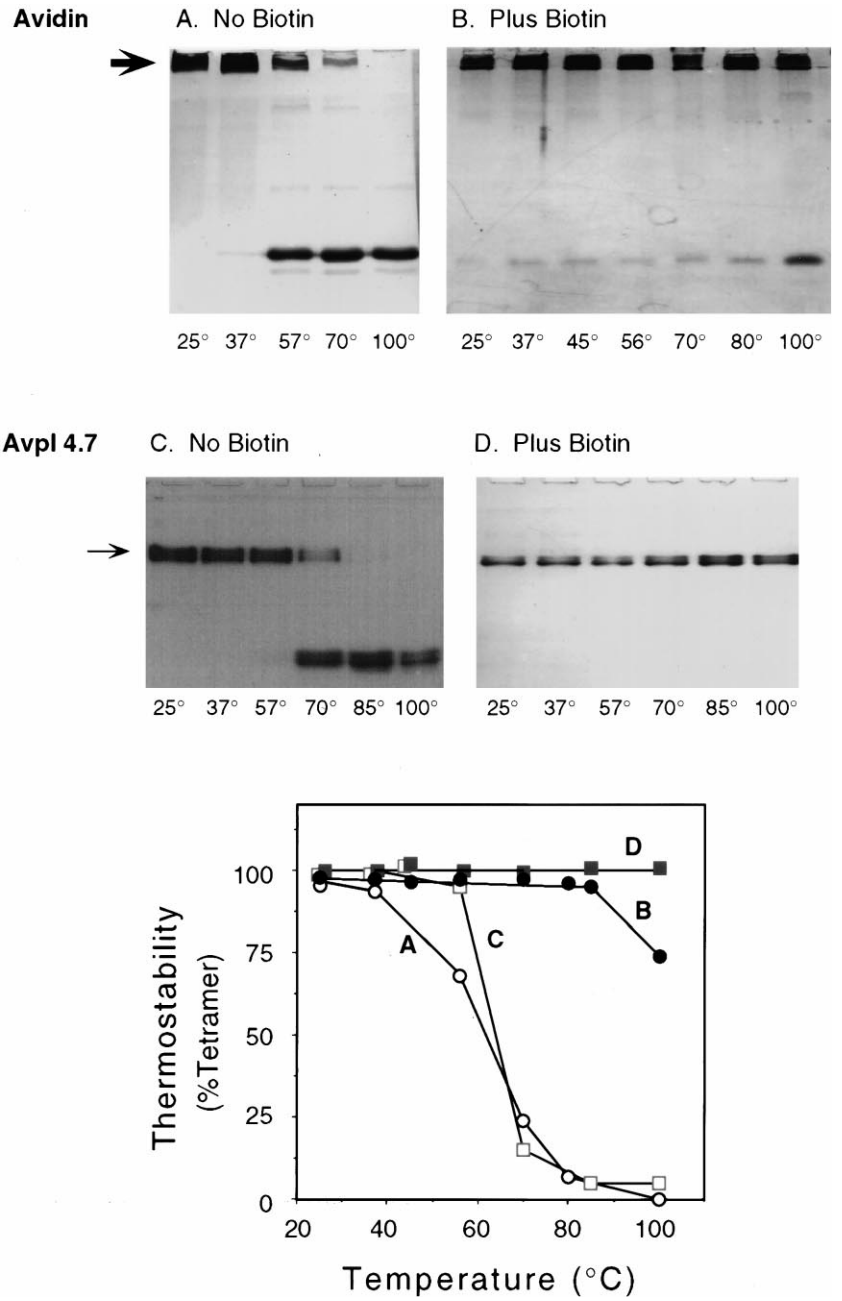

Fig. 3. Temperature-dependent dissociation of native avidin, AvdpI4.7 and their complexes with biotin. Samples of biotin-free and biotin-saturated avidin or AvdpI4.7 were combined with sample buffer and incubated for 20 min at the designated temperatures. The samples were then subjected to SDS-PAGE in 15% separating gels, and the gels were stained using Coomassie brilliant blue. Note that AvdpI4.7 tetramers (C and D, thin arrow) penetrated the upper gel, whereas those of the native avidin (A and B, thick arrow) failed to do so. Densitometry tracings from each of the gels were graphed as a function of temperature: A: avidin; B: avidin+biotin; C: AvdpI4.7; D: AvdpI4.7+biotin.

stances. The high p*I* of avidin has also been a detriment to its use in affinity-based drug targeting, since the positive charge of avidin is considered to be one of the major reasons for its rapid removal from the circulation system [3,19–21].

Site-directed mutagenesis is currently the method of choice to modify properties of a protein or to study the connection between its structure and function. Successful protein engineering requires understanding of the basic concepts of protein biosynthesis and structure. In the case of avidin, the availability of crystallographic data [11,12] together with the sequence information from avidin-related proteins [10] and streptavidin [9] have made it possible to design such changes in an intelligent manner.

In the present work, we wanted to study whether the high p*I* of avidin can be reduced without losing its stability or high affinity for biotin. For this purpose we constructed a set of reduced charge mutants with isoelectric points ranging from 9.4 down to 4.7. Most of these mutations are based on natural selection and evolution, since they were designed from sequence-based comparison with streptavidin and avidin-related proteins. Therefore Arg-2 was changed to Ala and Lys-3 to Glu according to the sequence of streptavidin. Lys-9 was re-

placed with Glu according to the sequence of the avidin-related protein, AVR1. Arg-26 was modified to Asn according to AVR1 and AVR2, and Arg-59 to Ala, since alanine appears at this position in every AVR protein. In AvdpI7.2, Lys-9 was replaced by Asp instead of Glu due to a PCR error. On the other hand, Arg-122 and Arg-124 were both altered to Ala, because these C-terminal residues were not observed in the crystal structure of avidin [11].

The biotin-binding properties of all charge mutants were similar to those of native avidin, according to the biosensor data. The actual affinities for biotin could not be determined, since it was essentially impossible to remove the bound proteins from the biotin-derivatized cuvette. This was not surprising, since the current biosensor technology can be used to determine affinities only up to $K_a \sim 10^{12}$ M^{-1}. We therefore decided to use 2-iminobiotin as a ligand. Chilkoti et al. [22] have previously demonstrated 2-iminobiotin to be a good reporter for intrinsic streptavidin-biotin interactions and the same is likely to hold true for avidin. The affinities of mutants for 2-iminobiotin (Table 1) were comparable to native avidin, which suggested that their affinities for biotin were also similar (K_as probably in the neighborhood of 10^{13}–10^{15} M^{-1}). This was not so surprising because all the changed amino acids were on the surface of the protein. In this regard, it has been shown previously that, at least in the case of lysozyme, the structural changes resulting from mutations of surface residues are smaller and localized near the mutation, whereas those involving buried residues are larger and may be transmitted to other parts of the protein [23].

As reported earlier [18], native avidin failed to penetrate the separating gel during non-denaturing SDS-PAGE and remained in the aggregated state in the stacking gel (Fig. 3A, arrow). This phenomenon was attributed to the high p*I* of avidin and possible strong electrostatic interactions with SDS. It is thus interesting to note that, under similar non-denaturing conditions, AvdpI4.7 (Fig. 3C) and most of the other reduced charge mutants migrated in the separating gel in a manner consistent with that of a tetramer.

When the thermal stability of the charge mutants was studied, it was observed that AvdpI9.4, AvdpI9.0 and AvdpI4.7 displayed very similar dissociation profiles to that of the wild-type avidin. In contrast, AvdpI7.9, AvdpI7.2 and AvdpI5.9 showed some differences. In the absence of biotin, the latter derivatives tended to dissociate into monomers already at room temperatures when SDS was present. Interestingly, all of the mutants were stabilized by the presence of biotin; the same mutants required temperatures up to 100°C to undergo denaturation as does wild-type avidin. AvdpI7.9, AvdpI7.2 and AvdpI5.9 all have mutations on Lys-3 and Lys-9 which suggests that either of these lysines (or both) could have some role in the stability of the avidin tetramer. On the other hand, AvdpI4.7 also bears both of these mutations, yet it seemed to be even slightly more stable than wild-type avidin. One possibility is that the additional mutations in AvdpI4.7 (Arg-26 to Asn-26 and Arg-59 to Ala-59) somehow counteract the effect of mutations on Lys-3/Lys-9. The reasons for this enigma remain to be investigated further.

In summary, we have constructed a progressive series of reduced charge mutants of the egg-white protein avidin, with p*I*s ranging from 9.4 to 4.7. All these mutant proteins bound biotin very strongly, and their thermostability was, in most cases, comparable to that of the wild-type avidin. The mutants also displayed reduced non-specific binding characteristics to DNA when compared to wild-type avidin. Our results demonstrate that the charge properties of avidin can be modified by protein engineering without disturbing the crucial biotin-binding activity. These modified avidins (especially AvdpI4.7) should also prove to be valuable in many applications of avidin-biotin technology, including affinity-based drug targeting and different separation technologies.

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