Recombinant NeutraLite Avidin: a non-glycosylated, acidic mutant of chicken avidin that exhibits high affinity for biotin and low non-specific binding properties

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Abstract A recombinant non-glycosylated and acidic form of avidin was designed and expressed in soluble form in baculovirus-infected insect cells. The mutations were based on the same principles that guided the design of the chemically and enzymatically modified avidin derivative, known as NeutraLite Avidin. In this novel recombinant avidin derivative, five out of the eight arginine residues were replaced with neutral amino acids, and two of the lysine residues were replaced by glutamic acid. In addition, the carbohydrate-bearing asparagine-17 residue was altered to an isoleucine, according to the known sequences of avidin-related genes. The resultant mutant protein, termed recombinant NeutraLite Avidin, exhibited superior properties compared to those of avidin, streptavidin and the conventional NeutraLite Avidin, prepared by chemo-enzymatic means. In this context, the recombinant mutant is a single molecular species, which possesses strong biotin-binding characteristics. Due to its acidic p*I*, it is relatively free from non-specific binding to DNA and cells. The recombinant NeutraLite Avidin retains seven lysines per subunit, which are available for further conjugation and derivatization.

Key words: Avidin-biotin technology; Non-glycosylated mutant; Non-specific binding; Protein engineering

1. Introduction

Avidin is a tetrameric glycoprotein isolated from chicken egg white. The ability of avidin to bind biotin with exceptionally high affinity ($K_d \sim 10^{-15}$M) [1] has been the basis for its exploitation as a molecular tool in biotechnological, diagnostic and therapeutic applications, collectively known as avidin-biotin technology [2–4]. The avidin tetramer consists of four identical subunits, all bearing 128 amino acids and possessing one biotin-binding site [5]. The protein is basically charged (p*I* ~ 10.5), and each of its monomers possesses eight arginine and nine lysine residues [5]. The polypeptide chain of avidin also contains a glycosylation site at residue Asn-17. The carbohydrate moiety accounts for about 10% of the molecular mass of avidin and exhibits extensive glycan microheterogeneity [6].

Despite the utility of chicken avidin in the many applications of avidin-biotin technology, there are some drawbacks associated with its use. Its high p*I* and the presence of carbohydrate can cause non-specific binding to extraneous material in certain applications, and these properties, therefore, hinder its use. Due to these difficulties, streptavidin, a non-glycosylated and neutrally charged bacterial counterpart of avidin [7], has virtually replaced avidin in these applications, even though avidin contains more lysine residues for potential attachment of probes, is more hydrophilic, and is considerably more abundant and cheaper than streptavidin.

In our attempts to improve the physicochemical properties of chicken avidin, we have shown earlier, that the oligosaccharide moiety can be removed enzymatically [8–10]. The resultant deglycosylated product can recognize biotin with high affinity constant, similar to that of the native glycoprotein. On the basis of the latter finding, the non-glycosylated and neutral avidin derivative, called NeutraLite Avidin (Belovo Chemicals, Bastogne, Belgium) or NeutrAvidin (Pierce, Rockford, IL, USA) was developed.

Using protein engineering techniques, we further demonstrated in a recent study [11] that the high p*I* of avidin can be reduced down to 4.7 by replacing arginines and lysines with neutral or acidic amino acids. The resultant mutant retained most of its lysine residues intact and available for further derivatization with, or attachment to, desired probes. Moreover, the biotin-binding capacity and stability of the avidin tetramer were maintained.

In the present work, we first modified by genetic means the glycosylation site of avidin, in order to produce an avidin mutant without the oligosaccharide moiety. It was especially of interest to determine whether removal of the sugar moiety by protein engineering would affect the biotin-binding capacity or the stability properties of the non-glycosylated avidin mutant. Secondly, we wanted to combine this sugarless mutant with the previously published charge mutant, Avm-p*I*4.7 [11], to further improve the non-specific binding characteristics of avidin. The ensuing mutant avidins, expressed in baculovirus-infected insect cells, displayed biotin-binding activity comparable with that of the wild-type avidin. Moreover, the combined mutant, ngAvm-p*I*4.7, showed clearly reduced non-specific binding to DNA and different cells, and therefore this mutant form of chicken avidin is particularly appropriate for general application in avidin-biotin technology.

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2. Materials and methods

2.1. Site-directed mutagenesis and preparation of avidin mutants

Mutagenesis of avidin cDNA [12] was accomplished by the PCR-based megaprimer method using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) as described earlier [11]. Infection of Sf 9 insect cells and purification of avidin mutants by affinity chromatography on 2-iminobiotin-agarose were also reported earlier [11,13].

2.2. Protein analyses

Several of the comparative assays of avidin and mutants used in this work have been described in earlier publications. The quaternary status of avidin was analyzed by FPLC on a recently calibrated Superose 12 column (Pharmacia) using an LKB HPLC system [14]. A sample (40 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. Binding kinetics were measured in a 2-iminobiotin cuvette, using optical biosensor technology (IASys Manual+, Affinity Sensors, Cambridge, UK) [11]. The thermal stability characteristics of the avidin mutants were assessed electrophoretically as described previously [15]. Protease sensitivity was determined according to Laitinen et al. [14]. Electrophoretic analysis was carried out using 15% (w/v) SDS–PAGE in a discontinuous buffer system [15,16].

2.3. Non-specific binding assay

Non-specific binding of avidin and mutants was examined by slot-blot assay using a Bio-dot® SF microfiltration apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Successive dilutions of salmon-sperm DNA (10, 3, 1 and 0.3 μg in 50 μl of 2×SSC solution) were fixed to the slots according to the manufacturer's instructions. The dried strips were quenched using 5×Denhart's solution. The desired avidin or mutant sample (25 μg in 1 ml of 5×Denhart's solution per 5 cm^2 nitrocellulose strip) was then added and incubated at room temperature for 90 min. The strips were then washed with PBS/0.05% Tween 20 solution, quenched with 0.5% BSA, and stained immunochemically as described earlier [13].

The test for non-specific binding to different cells was performed as follows. The cells chosen for this work were human platelets and lymphocytes, mouse hepatocytes, and *Escherichia coli* strain HB101, grown to the early logarithmic phase. Cell samples (~2.5 μg protein) were introduced into the slots. The cell suspension was allowed to dry by applying gentle vacuum, thus fixing the cells to the nitrocellulose membranes. The slot blots were quenched using 0.5% BSA, challenged by the desired avidin sample (2.5 μg in 50 μl PBS per slot). In both previous steps, the solutions were allowed to interact for 30 min prior to their evacuation. In between all steps, the blots were washed extensively using PBS/0.05% Tween 20 solution. The nitrocellulose membrane was removed from the apparatus, quenched again, and the bound avidin or derivative was detected immunochemically [13].

3. Results

3.1. Design of avidin mutants

We have recently reported the construction of fully functional avidin mutants, which exhibit p*I* values ranging from 9.4 down to 4.7 [11]. The mutations were devised by rational design, using sequence comparisons of streptavidin [17], the recently cloned avidin-related (*avr*) genes [18,19] and observations of the crystallographic structure of avidin [20–22]. By using the same general concept, we have now modified the glycosylation site of the avidin polypeptide chain, in order to produce a recombinant non-glycosylated avidin (ngAvm). This was accomplished by site-directed mutagenesis of Asn-17 to Ile. Isoleucine was chosen rather than alanine, because all avr proteins have Ile at this position (Fig. 1) and they all bind biotin strongly (K.J. Airenne, unpublished results). To further modify the properties of avidin we also combined the sugarless mutant ngAvm with the previously published charge mutant Avm-p*I*4.7 [11]. Of the six different reduced charge mutants reported in that study, Avm-p*I*4.7 was selected, since it displayed very tight biotin binding properties and was shown to be very stable. A list of the mutants used in this study is given in Table 1.

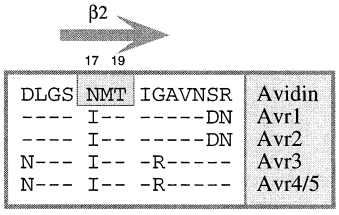

Fig. 1. Sequence alignment of the oligosaccharide-linkage site of native avidin and avidin-related proteins. The oligosaccharide linkage site of the native chicken avidin sequence is shaded and positioned in the middle of the second β-strand (indicated by the arrow). Note: all of the avr's bear an isoleucine at position 17, instead of the asparagine residue of the native avidin.

3.2. Production and purification of mutant avidins

Our previous results have shown that both avidin [13] and avidin mutants [11,14] can be efficiently produced in Sf9 insect cells, using a baculovirus expression system. Indeed, the expression of non-glycosylated avidin, ngAvm, was comparable to that of the wild-type avidin, although its secretion into the culture medium was clearly diminished (data not shown). The same phenomenon was also observed with the acidic, non-glycosylated derivative, ngAvm-p*I*4.7. Affinity chromatography on 2-iminobiotin agarose was used to purify the mutant avidins in one step to 95% homogeneity, as judged from SDS–PAGE. In the production of these proteins, a biotin-free culture medium was used, since the presence of biotin in the medium hinders subsequent affinity purification by blocking the binding sites of avidin and its mutants. The final yields of the mutant avidins were between 7 and 9 mg per liter culture.

3.3. Protein chemical analysis

As previously reported [15], at low temperatures wild-type avidin formed aggregates and failed to penetrate the separating gel in SDS–PAGE analysis (Fig. 2). This aggregation is presumably caused by the interaction of the negatively charged detergent with the positively charged tetrameric pro-

Table 1
Mutant avidins used in this study

Mutant	Description	Mutations
Avm-p*I*4.7	Recombinant acidic mutant of chicken avidin, having seven mutated residues (basic-to-neutral or basic-to-acidic) and exhibiting a p*I* of 4.7 [11].	R2A, K3E, K9E, R26N, R59A, R122A, R124A
ngAvm	Non-glycosylated mutant of avidin, carrying an asparagine-to-isoleucine mutation at residue 17.	N17I
ngAvm-p*I*4.7	A combined acidic, non-glycosylated mutant, containing all of the mutated residues carried by the above two mutants.	N17I, R2A, K3E, K9E, R26N, R59A, R122A, R124A

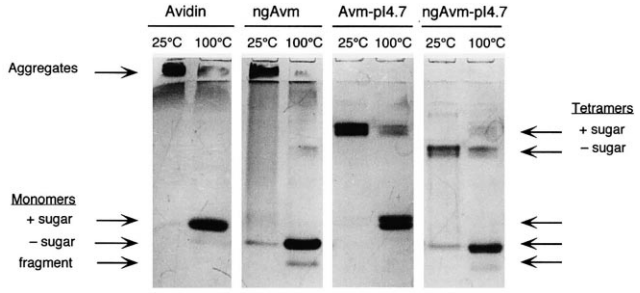

Fig. 2. SDS–PAGE of native and mutated avidins. Samples (1 mg/ml) of the native protein (Avidin), the N17I-mutated (non-glycosylated) derivative (ngAvm), the p*I* 4.7 mutant (Avm-p*I*4.7) and the combined non-glycosylated, p*I* 4.7 mutant (ngAvm-p*I*4.7) were diluted in SDS-containing sample buffer and subjected to non-denaturing (25°C) and denaturing (100°C) conditions prior to electrophoresis. Note, the native avidin and non-glycosylated mutant (i.e. the basically charged proteins) fail to penetrate the gel under non-denaturing conditions, whereas the acidic mutants migrate according to their expected molecular weight values.

tein. At higher temperatures, the aggregates dissociate, and the protein penetrates the gel as a monomer. The same phenomenon was observed with ngAvm, but, interestingly, under similar non-denaturing conditions, both acidic mutants (Avm-p*I*4.7 and ngAvm-p*I*4.7) migrated in the separating gel in a manner consistent with that of the tetramer.

The quaternary status of the ngAvm, Avm-p*I*4.7 and ngAvm-p*I*4.7, 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 showed that they behaved similar to wild-type avidin forming stable tetramers.

3.4. Biotin-binding activity

We have previously reported that the biotin-binding activity of Avm-p*I*4.7 was similar to that of the wild-type avidin [11]. It was also clear from the purification results, that ngAvm and ngAvm-p*I*4.7 both bound strongly to 2-iminobiotin, since they were efficiently affinity purified using this biotin derivative as a capturing ligand. An optical biosensor (IASyS Manual+) was used to further analyze the biotin-binding activities of ngAvm and ngAvm-p*I*4.7. However, the binding of these proteins to immobilized biotin was so strong that practically no dissociation was observed (data not shown). This already indicated that ngAvm and ngAvm-p*I*4.7 displayed very high affinity for biotin. In order to obtain more precise data on possible differences in binding affinities, 2-iminobiotin was used as a ligand instead of biotin. 2-Iminobiotin is a biotin analog, which binds avidin reversibly in a pH-dependent manner under relatively mild conditions [23]. The analysis of the binding curves of ngAvm, Avm-p*I*4.7 and ngAvm-p*I*4.7 showed that these mutations had only a nominal affect on the kinetic parameters of binding to 2-iminobiotin when compared to wild-type avidin (Table 2). The calculated k_{on} and k_{off} values (and, consequently, the K_D values as well) for these three mutants were found to be similar to those of wild-type avidin, suggesting that their biotin-binding activity was relatively unaffected by the changes in the amino acid sequence.

Table 2
Kinetic parameters for binding of avidin and different mutants to 2-iminobiotin at pH 9.5

Protein	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)
Avidin	1.6×10^{4}	3.1×10^{-4}	2.0×10^{-8}
Avm-p*I*4.7	1.6×10^{4}	2.0×10^{-4}	1.3×10^{-8}
ngAvm	1.9×10^{4}	6.6×10^{-4}	3.5×10^{-8}
ngAvm-p*I*4.7	3.3×10^{4}	7.1×10^{-4}	2.2×10^{-8}

3.5. Stability of avidin mutants

The thermal stability of ngAvm and ngAvm-p*I*4.7 was compared with that of the wild-type avidin by SDS–PAGE assay [15]. For this purpose the proteins were diluted in SDS-containing buffer and incubated at temperatures between 25°C and 100°C in the absence and presence of biotin, and the dissociation of the tetrameric to the monomeric form was observed. The ratio of tetramer to monomer was quantified by analyzing the densitometry tracings of the individual lanes and by plotting these values as a function of temperature (Fig. 3). In the absence of biotin, the tetramer-to-monomer transition of wild-type avidin occurred over a temperature range between 50°C and 70°C. On the other hand, the binding of biotin further stabilized the native protein so that temperatures near 100°C were required for dissociation to begin. The thermal stability properties of ngAvm were fully comparable with that of the wild-type avidin both with and without biotin. As previously reported [11], the Avm-p*I*4.7 also displayed very similar thermal stability characteristics when compared to that of the wild-type avidin. The ngAvm-p*I*4.7 was also found to be a very stable protein, although it exhibited slightly diminished thermostability both in the presence and in the absence of biotin.

To further investigate the stability of the avidin mutants, we tested their sensitivity to proteolysis by using a proteinase K

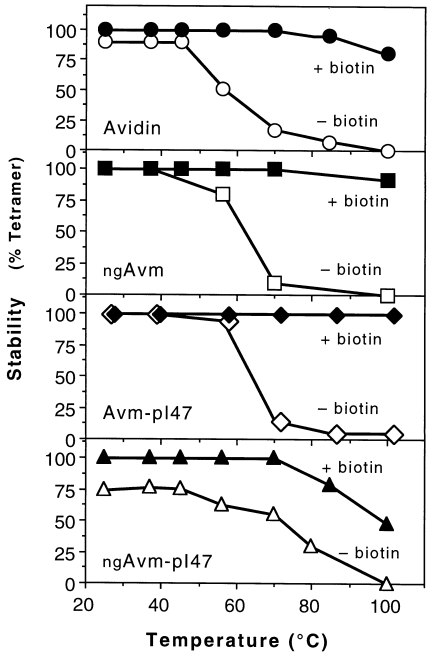

Fig. 3. Temperature-dependent dissociation of native and mutated avidins and their complexes with biotin. Samples of biotin-free and biotin-saturated avidin or avidin mutant were combined with sample buffer, incubated for 20 min at the designated temperatures, and subjected to SDS–PAGE in 15% separating gels. Densitometry tracings from each of the Coomassie-stained gels were graphed as a function of temperature.

assay system. It has been previously reported [24] that apoavidin is susceptible to slow limited proteolysis by proteinase K, whereas holoavidin is stable even in the presence of high amounts of the enzyme. Avidin and its mutants were thus incubated with proteinase K at 37°C, and samples were taken at predetermined time intervals. The samples were then subjected to SDS–PAGE, and the amount of intact protein in each lane was determined by densitometry and compared to that of an untreated control sample. The results were graphed as stability to protease treatment vs. time of the reaction (Fig. 4). As expected, without biotin the wild-type avidin was susceptible to slow proteolysis, but in the presence of biotin the native glycoprotein was stable. Likewise, the glycosylated mutant (Avm-p*I*4.7) displayed very similar behavior to wild-type avidin. In contrast, both non-glycosylated proteins (ngAvm and ngAvm-p*I*4.7) were clearly less stable in the absence of biotin. The binding of biotin, however, stabilized these proteins and rendered them resistant to proteinase K.

3.6. Non-specific binding characteristics of avidin mutants

For the present study, a slot-blot assay was developed to investigate the non-specific binding characteristics of avidin and its mutants to DNA and to various representative cell types (Fig. 5). Wild-type avidin bound strongly to DNA as expected. Most of this binding seemed to be charge-related, since there was a clear correlation between lowering the isoelectric point and reduced binding to DNA. Reducing the positive charge of avidin also drastically lowered the binding to all four different cell types used in this study, although Avm-p*I*4.7 still exhibited low levels of non-specific binding to certain cell types (i.e. hepatocytes and lymphocytes). As expected, ngAvm also bound strongly to DNA and to the different cell types. Interestingly, the removal of sugar from Avm-p*I*4.7 (i.e. ngAvm-p*I*4.7) further decreased its binding to different cells, such that no binding could be detected, as was the case with streptavidin.

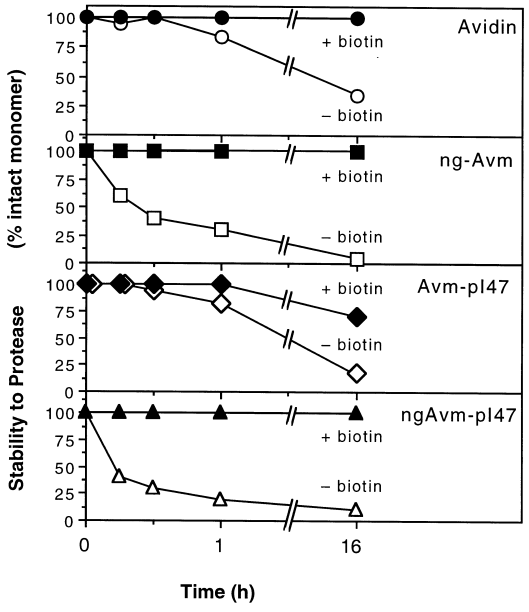

Fig. 4. Sensitivity of the native and mutant avidins to proteolytic digestion by proteinase K. The indicated protein, in the presence or absence of biotin, was mixed with a 1:50 ratio (w/w) of proteinase K to target protein, and samples were taken at the designated time intervals. The samples were dissolved in SDS-containing sample buffer, boiled for 10 min, and subjected to SDS–PAGE. The values represent the relative percent of intact monomer observed in the indicated sample, graphed as a function of time.

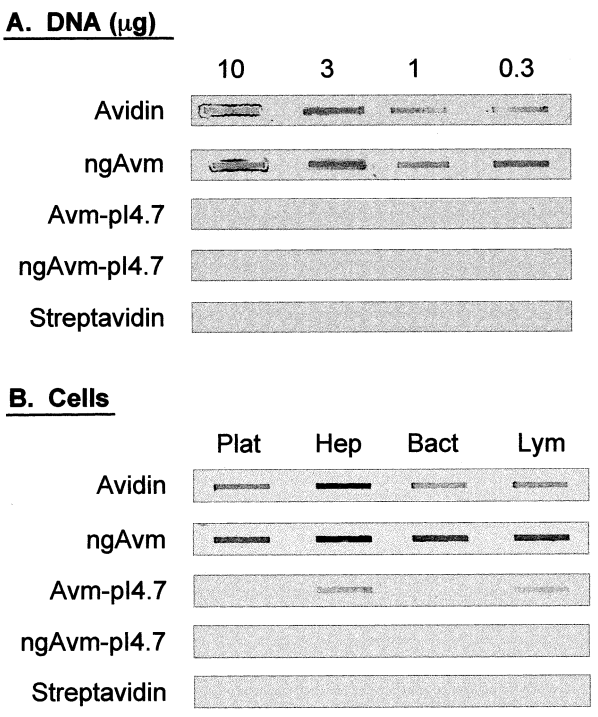

Fig. 5. Non-specific binding of native and mutant avidins to DNA and cells. The indicated amounts of salmon-sperm DNA or the indicated cell samples (2.5 μg protein per well) were fixed to nitrocellulose strips by slot blotting. Native or mutant avidin samples were applied, and the amount of avidin per slot was determined using anti-avidin antibody. Plat, platelets; Hep, hepatocytes; bact, *E. coli* cells; Lym, lymphocytes.

4. Discussion

Over the past two decades, the avidin-biotin system has been developed for general applications in the life sciences [2–4]. The technology has also been used recently in targeted drug imaging and as an agent for delivery systems in medicine [25–28]. However, the presence of sugar and the positive charge of avidin can be a hindrance to its use in these applications, due to non-specific binding to extraneous material.

In attempting to improve the properties of avidin, we have reported earlier [11] that its high p*I* can be reduced down to 4.7 by site-directed mutagenesis, without affecting the biotin-binding ability or the stability of the avidin tetramer. In the present study we mutated the glycosylation site of avidin in order to produce a non-glycosylated avidin and to explore whether the complete removal of the oligosaccharide moiety would generate deleterious effects on the physicochemical and/or biotin-binding properties of avidin. Moreover we combined this non-glycosylated avidin with a previously reported charge mutant of avidin (Avm-p*I*4.7) to create a mutated non-glycosylated avidin derivative (ngAvm-p*I*4.7) with additionally improved non-specific binding characteristics. Indeed, it has been previously shown [9] that enzymatically and chemically modified NeutraLite Avidin (deglycosylated and neutral) is

superior to native avidin in many biotechnological applications. However, it would be valuable to have a recombinant form of avidin that lacks the positive charge and oligosaccharide side chain, since the enzymatic and the chemical procedures used for the preparation of NeutraLite Avidin often lead to a mixture of products, which could affect biotin binding [29].

As in the case of the charge mutants [11], we again turned to nature for guidance in designing the non-glycosylated avidin, ngAvm. In addition to the avidin gene, the chicken genome includes at least five *avr*' genes which have recently been cloned by our group [18]. These *avr* genes were well conserved (overall identity over 90%) [19], and when expressed in insect cells showed strong binding to biotin (K.J. Airenne, unpublished results). Based on comparison of amino acid sequences of avidin and the avr proteins (Fig. 1), Asn-17 was replaced with isoleucine instead of the usual alanine, since all the avr proteins bear Ile in this position. It is interesting to note that all of the *avr*'s contain at least one alternative glycosylation site, i.e. comprising the Asn-Xaa-Ser/Thr sequence (not shown in the figure). These alternative sites, when modeled into the known three-dimensional structure of avidin, would be positioned on the surface of the molecule in close proximity to the position of Asn-17. Although the potential exists, it is currently unknown whether or not the avr proteins are indeed glycosylated in nature.

Based on the interaction analysis by optical biosensor, both the ngAvm and ngAvm-p*I*4.7 displayed similar biotin-binding properties to wild-type avidin. Both these proteins bound to biotin surface so tightly that practically no dissociation was detected; the current optical biosensor technology cannot be used to determine affinities stronger than $K_a \sim 10^{12}$ M. To further analyze the biotin-binding characteristics of ng-Avm and ngAvm-p*I*4.7, 2-iminobiotin surface was used, since avidin binds to 2-iminobiotin with lower, measurable affinity and 2-iminobiotin has been previously shown to be a good reporter for streptavidin-biotin interactions [30]. The kinetic parameters and the affinities of binding to 2-iminobiotin were in both cases similar to wild-type avidin (Table 2) demonstrating that neither the removal of sugar nor the reduction of p*I* caused noticeable effects in biotin-binding ability of avidin.

Removal of the oligosaccharide moiety also had little effect on the thermal stability characteristics of avidin, since ngAvm was found to be as stable as the wild-type avidin. The ngAvm-p*I*4.7 displayed slightly decreased thermostability properties, but when bound to biotin it still required temperatures over 80°C to dissociate (and only partially). Interestingly when susceptibility to proteinase K was tested, it was observed that without biotin both ngAvm and ngAvm-p*I*4.7 were clearly more sensitive to limited proteolysis than either wild-type avidin or Avm-p*I*4.7. However, biotin stabilized all these proteins and rendered them resistant to proteolysis. It has been previously shown [24] that in the case of apoavidin, proteolysis with proteinase K results in limited attack at the loop between β-strands 3 and 4 (the specific sites of proteolysis are at Thr40-Ser41 and Asn42-Glu43), leaving other parts of the molecule intact. According to the three-dimensional structure [20,31], this loop is flexible and accessible to solvent in apoavidin, but when biotin is bound it becomes ordered and locks the biotin in the binding site. This behavior of the 3-4 loop is the most likely explanation for resistance of holoavidin to proteinase K. Analysis of the three-dimensional structure of avidin also revealed that the glycosylation site, Asn-17, is located near the 3-4 loop. It is therefore plausible that the sugar moiety would protect this loop from proteolysis, since ngAvm and ngAvm-p*I*4.7 are both degraded at a substantially faster rate than either wild-type avidin or Avm-p*I*4.7.

In a recent article [32], an acidic mutant of chicken avidin was expressed in *E. coli*. Despite the presence of a carbohydrate-attachment site (Asn16-Met17-Thr18), the gene product was non-glycosylated as would be expected in a bacterial host cell system. One of the interesting characteristics of this particular recombinant acidic avidin was its relatively low response to anti-avidin antibodies, to which the authors attributed an altered antigenicity. An alternative explanation for the low response to antibodies would be that the acidic mutant avidin has reduced non-specific binding properties, resulting in a comparatively low signal when compared to that of the native (basically charged) chicken avidin. Indeed, all of the avidin mutants produced in our laboratory proved to be highly antigenic in properly controlled experiments (Tikva Kulik, unpublished results).

In summary, we have produced a non-glycosylated avidin mutant, ngAvm, which bound biotin very tightly and displayed thermostability characteristics, similar to those of the wild-type avidin. These results support the earlier claims [8–10] that the sugar side-chain is not important for biotin-binding or for maintaining the high thermostability properties of native avidin. Secondly, we have combined ngAvm with an acidic mutant, Avm-p*I*4.7 [11], to further enhance the properties of avidin for general application in avidin-biotin technology. The resultant non-glycosylated, acidic mutant, ngAvm-p*I*4.7, was found to be very stable and to exhibit biotin-binding activity comparable to that of wild-type avidin. Moreover, ngAvm-p*I*4.7 displayed clearly reduced non-specific binding both to DNA and to different cell types. This mutant could therefore be particularly useful in future localization and separation studies where high background is a problem. Furthermore, the non-glycosylated, acidic avidin may also be applicable in affinity-based drug targeting. In this regard, chicken avidin would be preferable to streptavidin for in vivo studies. However, one of the problems experienced in using avidin is its rapid removal from the circulation system. The high p*I* and sugar moiety of avidin have been considered as the main reasons for its rapid clearance [29,33–35], and the mutant equivalent to NeutraLite Avidin described herein may provide an elegant solution to this problem.

Finally, although *E. coli* expression systems are cheaper and more efficient in most cases when glycosylation is not needed, production of the avidin mutants in baculovirus-infected insect cells results in a soluble gene product. This is clearly advantageous over renatured recombinant avidin and streptavidin mutants produced in *E. coli*. In addition to the baculovirus production system used in this study, mutated non-glycosylated avidin genes could also be attractive topics for production in other eukaryotic systems like yeast, fungi or plants. Indeed, it has been shown that transgenic corn can produce high amounts of wild-type avidin [36], and it would be interesting to apply such a system for the production of large quantities of non-glycosylated avidin mutants.

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