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Mutation of a critical tryptophan to lysine in avidin or streptavidin may explain why sea urchin fibropellin adopts an avidin-like domain

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Abstract **Sea urchin fibropellins are epidermal growth factor homologues that harbor a C-terminal domain, similar in sequence to hen egg-white avidin and bacterial streptavidin. The fibropellin sequence was used as a conceptual template for mutation of designated conserved tryptophan residues in the biotin-binding sites of the tetrameric proteins, avidin and streptavidin. Three different mutations of avidin, Trp-110-Lys, Trp-70-Arg and the double mutant, were expressed in a baculovirus-infected insect cell system. A mutant of streptavidin, Trp-120-Lys, was similarly expressed. The homologous tryptophan to lysine (W→K) mutations of avidin and streptavidin were both capable of binding biotin and biotinylated material. Their affinity for the vitamin was, however, significantly reduced: from $K_d \sim 10^{-15}$ M of the wild-type tetramer down to $K_d \sim 10^{-8}$ M for both W→K mutants. In fact, their binding to immobilized biotin matrices could be reversed by the presence of free biotin. The Trp-70-Arg mutant of avidin bound biotin very poorly and the double mutant (which emulates the fibropellin domain) failed to bind biotin at all. Using a gel filtration fast-protein liquid chromatography assay, both W→K mutants were found to form stable dimers in solution. These findings may indicate that mimicry in the nature of the avidin sequence and fold by the fibropellins is not designed to generate biotin-binding, but may serve to secure an appropriate structure for facilitating dimerization.**

Key words: Avidin-biotin technology; Recombinant avidin and streptavidin; Functional dimer; Biotin-binding; Reversible

1. Introduction

Chicken egg-white avidin and bacterial streptavidin share a similar high affinity for the vitamin biotin ($K_d \sim 10^{-15}$ M), although their primary structures are not well-conserved (~30% identity and 41% similarity) [1]. In spite of the relatively low sequence homology, the two proteins share the same tertiary fold, similar tetrameric quaternary structures and a nearly identical arrangement of amino acids within their respective biotin-binding pockets [1–4].

A family of other proteins exists, the fibropellins, which harbor a C-terminal domain similar in sequence to avidin and streptavidin [5,6]. Fibropellins are epidermal growth factor (EGF) homologues found in the hyaline layer of the extracellular matrix in sea urchin embryos. The fibropellins of interest to our studies consist of repeated EGF-like N-terminal domains and, unexpectedly, a single avidin-like C-terminal domain. However, neither the function nor the three-dimensional structure of the avidin-like domain is known. It is not even known whether the domain is capable of binding biotin. Comparison of the amino acid residues of the fibropellins, which are homologous to the biotin-binding residues of avidin and streptavidin, revealed that most of them appeared to be conserved. The most remarkable alteration is the replacement of Trp-110 in avidin (equivalent to Trp-120 in streptavidin) with Lys in all four fibropellins [7].

In our previous studies on the structure-function relationship in the avidin/streptavidin-biotin system, we have employed a variety of different approaches to examine how flexible one can be in modifying different amino acids in the binding site while still conserving the property of biotin-binding. These include chemical modification studies [8–12], X-ray analysis [1,13] and sequence comparisons with naturally occurring, biotin-binding proteins, e.g. different types of streptavidins [14] and avidin-related proteins [15]. The results indicated that there is little compromise regarding the types and positions of binding site residues. Most of them are strictly conserved, in order to maintain the property of strong binding to biotin. On the other hand, considerable flexibility can be applied in modifying residues outside of the binding site. In this regard, only about 30% of the primary structure is conserved between avidin and streptavidin [1].

As structural and potentially functional entities, both the avidin and streptavidin tetramer can conceivably be divided into three different types of dimer pairs [1]. 'Functional' dimers would presumably be formed between monomers 1 and 2 and between monomers 3 and 4, numbered according to Livnah et al. [1], wherein the conserved Trp-110 of one avidin monomer (Trp-120 in streptavidin) is part of the biotin-binding pocket of its affiliate and vice versa. 'Structural' dimers were likely formed between monomers 1 and 4 (or 2 and 3) in both proteins. Trp-110 (or Trp-120) is also noteworthy in another sense: this particular residue has a remarkable effect on the stability of the tetrameric proteins [1].

In view of the natural 'replacement' of Trp-110 to Lys in fibropellin, it was of interest to determine how such a replacement would affect the characteristics of biotin-binding and the quaternary structure of either avidin or streptavidin. Our findings indicate that such a mutation results in a stable dimer in both proteins, which exhibit reversible biotin-binding properties.

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2. Materials and methods

2.1. Materials

Biotin-agarose and 2-iminobiotin-agarose were purchased from Sigma Chemical (St. Louis, MO, USA). Egg-white avidin was a gift of Belovo Chemicals (Bastogne, Belgium) or STC (Winnipeg, Man., Canada). Nitro-avidin was prepared as previously described [16]. Streptavidin was provided by S.p.a. (Milan, Italy). Rabbit avidin and streptavidin antibodies were acquired from DAKO A/S (Glostrup, Denmark). Goat anti-rabbit immunoglobulin G (IgG) alkaline phosphate conjugate and the low-range sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA, USA). JM109 and DH10Bac *Escherichia coli* strains were used in cloning procedures. Mutants were produced in baculovirus-infected Sf9 insect cells.

2.2. Recombinant bacmids and baculoviruses

Multiple sequence alignment (not shown) of avidin, streptavidin and sea urchin fibropellins was carried out using CUSTAL X [17] and the GCG package (Genetic Computer Group, Madison, WI, USA) was employed to calculate the theoretical molecular weights for the mutant proteins.

Avidin and streptavidin cDNAs were mutated by the megaprimer method [18], using pGEMAV as an avidin template [19] and the streptavidin gene from *S. avidinii*, prepared as previously described [20]. After a second PCR amplification, the product for the avidin mutant was digested with *Bgl*II and *Hin*dIII and the product for the streptavidin mutant was digested with *Bam*HI and *Hin*dIII. Both digests were then extracted from agarose and cloned into *Bam*HI/*Hin*dIII-treated pFASTBAC1 to construct the recombinant vectors, pFδ9 (avidin mutant Avm-W110K), pFδ10 (Avm-W70R), pFδ17 (Avm-W70R and W110K) and pFSAK (streptavidin mutant Savm-W120K). The resultant vectors were transformed into JM109 cells to construct the recombinant baculoviruses. The correct nucleotide sequence was confirmed in each case by dideoxynucleotide sequencing with an automated DNA sequencer. The preparations of the recombinant viruses were finally completed according to the manufacturer's instructions for the Bac-To-Bac Baculovirus Expression System (Gibco BRL, Life Technologies, Gaithersburg, MD, USA).

The primary virus stocks were amplified for large-scale production of the mutants (Avm-W110K, Avm-W70R, Avm-W70R and Savm-W120K) and the titers of virus stocks were determined by a plaque assay procedure [21].

2.3. Preparation of mutant proteins

Production of mutant proteins was carried out essentially as previously described for recombinant avidin [22], with the exception that the cell density in different experiments varied from 1×10^6/ml to 2×10^6/ml and the m.o.i. from 0.1 to 5. In experiments where mutant proteins were purified, infections were carried out with cells that were transferred to biotin-free medium (Gibco BRL, Cat. # 041-94100).

Purification of the mutant proteins from the corresponding cell extract was performed on 2-iminobiotin-agarose as reported by Airenne et al. [22]. Following cell lysis, the soluble fraction was brought to pH 11 and applied to a 2-iminobiotin-agarose column. The mutants were eluted using 50 mM ammonium acetate (pH 4/0.1 M NaCl). Later in the study, purification of Avm-W110K on a biotin-agarose column proved superior to that using 2-iminobiotin. In this case, the pH of the cell extract was not altered prior to application on the column. Adsorbed material was eluted either using 0.2 mg/ml biotin in 50 mM ammonium acetate (pH 4/0.1 M NaCl) or by 0.4 M acetic acid.

2.4. Biotin-binding assays

Biotin-binding was initially assessed by a dot blot assay, using a modification of procedures described in Bayer et al. [23] and Bayer and Wilchek [24]. Briefly, successive dilutions of biotinylated bovine serum albumin (BSA) were applied to dot blots. The blots were quenched using BSA and an avidin, streptavidin or mutant test sample (1 μl of a 20 μg/ml solution in phosphate-buffered saline (PBS)) was applied. The interaction was carried out at 23°C for 30 min. The blots were washed with PBS-0.5% (v/v) Tween solution and stained immunochemically as described below.

A complementary method was used to achieve defined affinity constants for the mutants. An IASyS optical biosensor (Affinity Sensors, Cambridge, UK) and commercially available biotin-aminosilane cuvette (FCB5401) were used to obtain the kinetic measurements of the interaction between Avm-W110K or Savm-W120K and biotin. Different concentrations of mutant protein were allowed to bind to the biotinylated surface and the affinity constants were calculated using the FASTfit software, developed by Affinity Sensors. Experiments were performed in PBS, containing 1 M NaCl, and cuvette regeneration was accomplished by 20 mM HCl.

2.5. Reversibility assays

2.5.1. Competitive biotin-binding enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 1 μg biotinylated BSA per well in 100 μl coating buffer (15 mM sodium carbonate buffer, pH 9.6). The plates were incubated overnight at 4°C (or, alternatively, at 37°C for 2 h), washed with PBS, quenched using 0.5% BSA in PBS at 37°C for 1 h and washed again in wash buffer A (1 M NaCl and 0.5% v/v Tween 20 in PBS). Three concentrations (10, 20 and 50 ng/well in assay buffer A, consisting of 0.5% BSA, 0.5% Tween 20 and 1 M NaCl in PBS) of mutant were applied in sextuplicate to the wells. Native avidin was used as a negative control (i.e. no reversibility) and nitro-avidin [16] as a positive control (normally ~70% reversibility). The wells were washed with wash buffer A.

At this point, the microtiter plate contained different types of avidin or streptavidin or mutant, bound through the biotin moiety of the adsorbed biotinylated BSA. The remainder of the assay was designed to test how much could be released using biotin.

Half of the appropriate wells (i.e. three wells each of the test samples) was incubated with 0.5 mM biotin dissolved in assay buffer A at 37°C for 1 h, in an attempt to displace, if possible, the adsorbed protein from the biotinylated BSA. The plates were then washed three times with wash buffer A and once with PBS alone.

The amounts of avidin, streptavidin, nitro-avidin or mutant remaining on the plates was determined immunochemically as described below. The percentage reversibility of the interaction between the desired biotin-binding protein and the biotin ligand was determined according to the following equation:

$$\text{Reversibility } (\%) = 100(A-B)/A$$

where A = the amount of protein bound to the wells in the initial interaction and B = the amount of protein remaining after subsequent interaction with free biotin.

2.5.2. Competitive biosensor technology. A complementary reversibility assay was also devised, which employed the optical biosensor (IASyS) and commercial biotin cuvettes as described above. In this case, avidin, streptavidin or their mutants were allowed to bind to the biotin-aminosilane cuvette in PBS, containing 1 M NaCl. After careful washing, the binding was measured and the binding buffer was saturated with biotin. The proteins were allowed to elute until the system reached an equilibrium level. After this, another extended wash was performed using the binding buffer. The residual amounts of bound protein, following the wash, were compared to those measured after the initial binding phase and reversibility was determined according to the above equation.

2.6. Immunoassay

Avidin- or streptavidin-containing dot blots or plates were incubated with rabbit anti-avidin or anti-streptavidin Ig at 37°C for 1 h. The antibody was diluted (1:5000 from a 10 mg/ml solution) in assay buffer B (0.5% BSA, 0.05% v/v Tween 20, 0.9% NaCl and 0.05% w/v sodium azide in 50 mM Tris-HCl buffer, pH 7.75) to prepare final concentrations of 3 μg/ml for nitrocellulose strips and 330 ng/well for microtiter plates. The plates were again washed three times with wash buffer B (0.05% v/v Tween 20 in 50 mM Tris-HCl buffer, pH 7.75) and then incubated with anti-rabbit IgG-conjugated alkaline phosphatase (Jackson ImmunoResearch) at 37°C for 1 h, diluted 1:5000 in assay buffer B. Following another three washes in wash buffer B and a single wash in PBS, substrate solution (10 mg *p*-nitrophenyl phosphate in 10 ml 0.1 M diethanolamine buffer, pH 9.8, containing 0.5 mM $MgCl_2$) was added. Color development (405 nm) was measured at 15 min intervals, using an EL309 Microplate Autoreader (Bio-Tek Instruments, Burlington, VT, USA).

2.7. Sensitivity to proteases

A sample (50 μl of a 1 mg/ml aqueous solution of avidin, strepta-

vidin or mutant in the presence or absence of excess free biotin) was added to an equal volume of 100 mM Tris-HCl buffer, pH 7.8. To this solution, proteinase K (5 μl of a 0.2 mg/ml aqueous solution) was added. The reaction solution was incubated at 37°C. At designated time intervals, 20 μl samples were taken and stored at −20°C. To each sample, 10 μl of sample buffer was added, the samples were boiled and subjected to SDS-PAGE on 18% gels. The amount of intact avidin in the expected band was determined in each sample by densitometry and compared to that of an untreated control sample (defined as 100%). The results were graphed as stability to protease treatment (sample/control × 100%) versus time of reaction.

2.8. Miscellaneous methods

SDS-PAGE and immunoblot analyses were as previously reported by Airenne et al. [22]. For thermostability analysis, the protein samples were combined with sample buffer and incubated at selected temperatures for 20 min before being subjected to SDS-PAGE [24] on 15% gels. The gels were stained using Coomassie brilliant blue.

Circular dichroism (CD) spectroscopy studies were carried out using a Jasco 715 circular dichrometer at the far-UV region. Measurements were performed in circular 1 mm cuvettes at 22°C in 100 mM potassium phosphate buffer, pH 7.4. Variance, caused by different concentrations, was corrected in final spectra.

The quaternary status of avidin, streptavidin or mutants was determined by fast-protein liquid chromatography (FPLC), performed on a Superose-12 column (Pharmacia) using a LKB HPLC system. Samples (40 μg in 100 μl phosphate buffer in 0.65 M NaCl, pH 7.2) were applied and chromatography was carried out at a flow rate of 0.5 ml/min, using the same buffer and ionic strength in the equilibration and running phases. The column was calibrated using bovine γ-globulin, BSA, an egg-white avidin standard, ovalbumin, carbonic anhydrase, ribonuclease and cytochrome *c* as molecular weight markers.

3. Results

3.1. Synthesis and purification of mutant proteins

Sf9 cells, infected with recombinant viruses, produced three separate protein bands in SDS-PAGE, which were detected by immunoblot analysis using avidin-specific antibody. The bands represent carbohydrate-free and two differently glycosylated avidin forms [22]. These bands were not observed from cells infected by wild-type virus (not shown). Cell lysates were clarified by centrifugation (1000 × *g*, room temperature, 5 min) and adjusted to pH 11/1 M NaCl before being subjected to purification on 2-iminobiotin-agarose. The efficiency of such columns was less than 25% for Avm-W110K. The residual fraction was therefore subjected to purification using a biotin-agarose column and the final combined yield surpassed 75%. The same high yield could also be achieved by purifying mutant samples with biotin-agarose alone. Efficient elution of the 2-iminobiotin-purified Avm-W110K was achieved using pH 4 buffer. A biotin-containing solution was effective for elution of the mutant protein from biotin-agarose. Elution using 0.4 M acetic acid was also possible but less efficient.

Savm-W120K was prepared by a similar procedure. Following SDS-PAGE and immunoblot analysis, only a single band, consistent with monomeric core streptavidin, was detected by streptavidin-specific antibody. The mutant protein was isolated on a 2-iminobiotin column and efficient elution of Savm-W120K was achieved using pH 4 buffer.

In contrast to successful purification of Avm-W110K and Savm-W120K, we were unable to purify two other avidin mutants, Avm-W70R and Avm-W70R and W110K, using 2-iminobiotin or biotin columns.

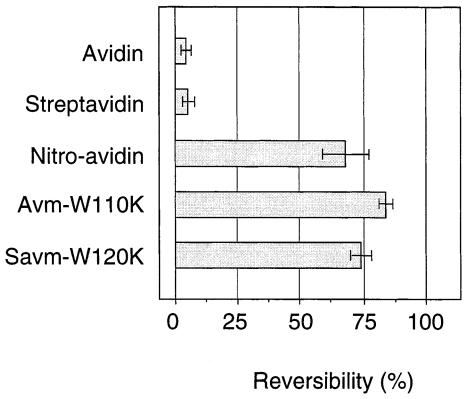

Fig. 1. Reversibility of biotin-binding activity of the W→K mutants of avidin and streptavidin. Both ELISA and IASyS protocols were used (see Section 2) and the results are expressed as % reversibility (mean ± S.E.M.). Avidin and streptavidin were used as negative (irreversible) controls and nitro-avidin was used as a positive control.

3.2. Biotin-binding properties

Dot blot assays revealed that both the purified Avm-W110K and Savm-W120K mutants bound biotin (not shown). In order to provide more quantitative data, the W→K mutants were subjected to interaction analysis using an IASyS apparatus and a biotin-aminosilane cuvette. Using this system, a measurable dissociation constant (K_d value) for the interaction between Avm-W110K and biotin was determined directly from the binding curves to be 2.8×10^{-8} M (Table 1). A very similar value (2.7×10^{-8} M) was also obtained from the equilibrium response data. The equivalent values for Savm-W120K were 5.8 and 5.9×10^{-8} M. Unlike biotin cuvettes adsorbed with native avidin, the mutant-adsorbed cuvettes could be regenerated completely by a 1 min wash with 20 mM HCl.

As shown in Fig. 1, the biotin-binding property of both W→K mutants was reversible. In fact, the extent of release of the mutant from the biotinylated surface was superior to that exhibited by nitro-avidin [16]. The results for reversibility of binding were similar for both of the assay techniques used in this study.

3.3. Structural and stability properties of Avm-W110K and Savm-W120K

The secondary structure of Avm-W110K was studied by

Table 1
Affinity constants between immobilized biotin and the mutants, determined using an IASyS optical biosensor

Mutant	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_d (M)[a]	K_d (M)[b]
Avm-W110K	1.08×10^{6}	0.0292	2.7×10^{-8}	2.8×10^{-8}
Savm-W120K	0.118×10^{6}	0.0071	6.0×10^{-8}	5.8×10^{-8}

[a]The dissociation constant (K_d) was calculated from the k_{ass} (association rate constant), derived from association analysis using the plot of K_{on} against the protein concentration, and the k_{diss} (dissociation rate constant), calculated directly from dissociation curves.
[b]The dissociation constant was determined directly from the binding curves.

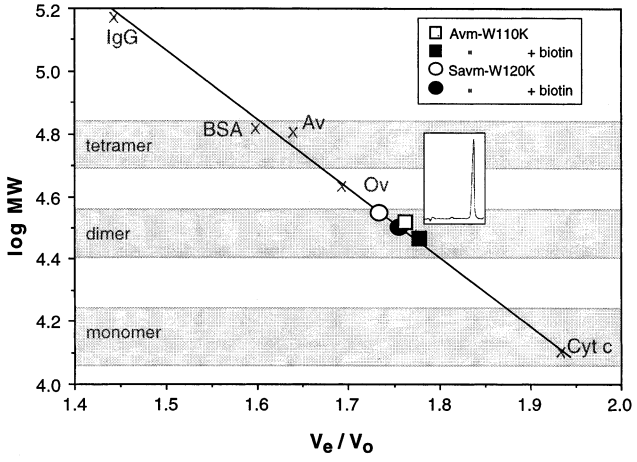

Fig. 2. FPLC gel filtration profile of the W→K mutants of avidin and streptavidin, in the presence and absence of free biotin. A commercial Superose-12 column was used for separation. Human Ig (IgG), BSA, avidin (Av), ovalbumin (Ov) and cytochrome *c* (Cyt c) were used as markers to calibrate the column. The inset shows the observed peak for Avm-W110K. Very similar, well-defined peaks were also obtained for Savm-W120K and for both mutants in the presence of biotin. The migration pattern of both mutants is consistent with a dimer, whether in the absence or presence of biotin.

CD spectroscopy. The far-UV spectra (data not shown) resembled that of the wild-type avidin [25], suggesting that the β-barrel fold of the avidin mutant had remained intact.

In order to determine the quaternary status of the W→K mutants, gel filtration FPLC was performed (Fig. 2). In each case, the mutant migrated as a single symmetrical peak (Fig. 2, inset). When compared to standards and a commercial avidin preparation, the molecular weight of the Avm-W110K mutant was determined to be 31 200 Da (average of three independent experiments). The calculated theoretical molecular weight of the monomer (without sugar) is 14 285 Da and the carbohydrate moiety comprises about 10% of the molecular mass [26,27]. According to these values, the best fit for the quaternary state of Avm-W110K is a dimer, the hypothetical molecular weight of which would be approximately 31 500 Da. The corresponding streptavidin mutant, Savm-W120K, gave a similar value of 36 700 Da, which is also consistent with a dimer. The presence of biotin failed to affect the apparent quaternary structures of either mutant. Both Avm-W110K and Savm-W120K behaved as dimers whether in the presence or in the absence of biotin. Thus, in each case, mutation of Trp-110 in avidin or the analogous residue (Trp-120) in streptavidin caused a conversion of the tetramer to a dimeric form of the mutant protein.

SDS-PAGE has previously been used to analyze the stability properties of avidin, streptavidin [22,28] and a progressive series of avidin charge mutants [29]. Using this procedure, both Avm-W110K and Savm-W120K were found to dissociate into monomers in the presence of SDS already at room temperature (Fig. 3). Unlike the native proteins and reduced charge mutants, the presence of biotin failed to stabilize the W→K mutants and tetramers were not observed. These results are consistent with the FPLC data, which demonstrated the dimeric nature of both mutant proteins. In both cases, the respective dimer is less stable than the native tetramer and the detergent appears to convert the dimer to the monomer.

The stability properties of both mutants were also studied by their susceptibility to proteinase K treatment. Compared to native avidin, Avm-W110K was extremely sensitive to protease treatment (Fig. 4). Within minutes, all of the avidin dimer was cleaved and the resultant lower-molecular weight bands were consistent with the previously described proteinase K-sensitive cleavage site in one of the loops [30]. Unlike native avidin, the presence of biotin failed to protect the mutant from rapid proteolytic cleavage. In contrast, the streptavidin mutant Savm-W120K, like the native protein, was completely stable to the action of proteinase K, both in the presence and in the absence of biotin.

Both W→K mutants of avidin and streptavidin were generally quite stable under normal conditions. One indication of such a stability of the Avm-W110K mutant is the observation that a sample, stored at room temperature in the absence of preservatives for 1 month, was unaltered in its biotin-binding properties, as measured by optical biosensor analysis (IASyS) as described above (not shown).

4. Discussion

The impetus and rationale behind this work emanates from the comparison of three different protein families, egg-white avidin, bacterial streptavidin and sea urchin fibropellin, which are moderately conserved in their primary amino acid sequences [6]. Two of these proteins, avidin and streptavidin, bind the vitamin biotin with the highest known biological interac-

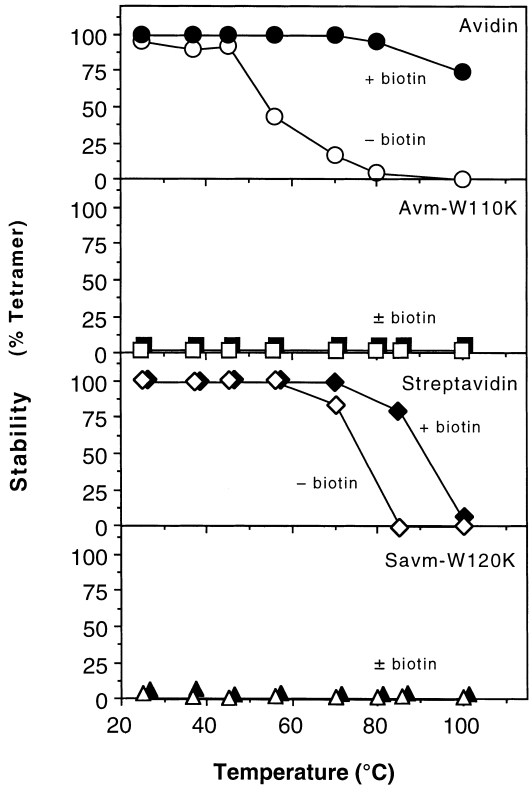

Fig. 3. Thermostability analysis of avidin, streptavidin and their W→K mutants in the presence of SDS. Samples in the presence or absence of free biotin were dissolved in SDS-containing sample buffer, incubated for 20 min at the indicated temperatures and subjected to SDS-PAGE. The ratio of tetramer to monomer was determined by densitometry tracings and the results were graphed as a function of the temperature [26].

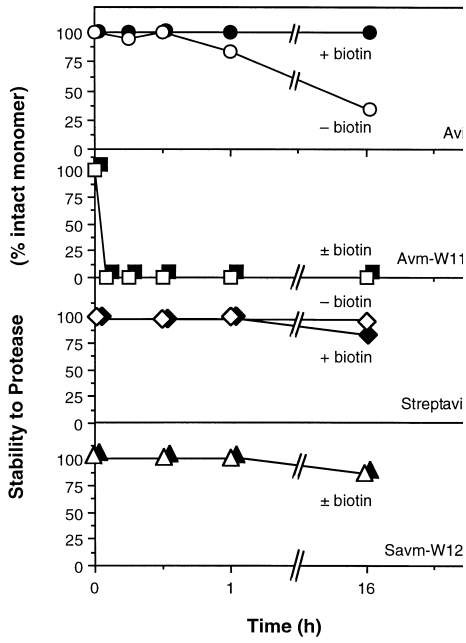

Fig. 4. Sensitivity of the W→K mutants of avidin and streptavidin to proteolytic digestion by proteinase K. The mutants or native proteins, in the presence or absence of biotin, were mixed with a 1:50 ratio (w/w) of proteinase K to target protein and samples were taken at the designated time intervals.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

tion between a protein and a small ligand [31]. Their three-dimensional structures have been determined and the residues of the protein which interact with biotin have been characterized [1–4]. In contrast, the structure and function of the avidin-like domain of the fibropellins are unknown and a recombinant form has yet to be expressed. Despite the striking resemblance of its primary structure to both avidin and streptavidin, it is not yet known whether fibropellins bind to biotin or not.

Most of the important biotin-binding residues in avidin and streptavidin are conserved in the fibropellins (Table 2). Several discrepancies, however, can be noted. For example, a binding site serine residue in both avidin and streptavidin, which forms a hydrogen bond with the carbonyl carbon of the biotin ureido ring [1–4], is substituted in the fibropellins by an aspartic acid. Aspartic acid, however, is also capable of forming a hydrogen bond and one could envision a conservative substitution in this case. Much more intriguing is the replacement of two tryptophans, which contribute to the aromatic cage of the binding pocket. One of these is replaced by an arginine and a second by a lysine. Using this information, we tried to replace either or both of these tryptophan residues in avidin to see whether the mutant proteins could be expressed in a functional form. Thus far, we were able to express all three mutants, i.e. Avm-W110K, Avm-W70R and the double mutant Avm-W70R and W110K. The latter, however, appeared to be inactive and failed to bind to either 2-iminobiotin-agarose or biotin-agarose. The Avm-W70R mutant appeared to exhibit relatively low binding to both columns, but we were as yet unable to prepare sufficient protein for biochemical analysis. Therefore, the present article concentrates on our successful cloning and expression of Avm-W110K. On the basis of the results obtained for this mutant, we also prepared and examined the cognate mutant from streptavidin, namely Savm-W120K.

It is perhaps fortunate that we had nature on our side to guide us in the choice of these two related mutations, since the replacement of a tryptophan by a lysine is clearly an unorthodox substitution. It is interesting that such an extraordinary mutation did not seem to interfere significantly with the expression and purification of the mutant proteins, a fact that probably reflects the remarkable stability of the native protein. Nevertheless, the W→K mutation had two major effects on the avidin and streptavidin molecules. In both proteins, stable dimers were generated and the affinity constant for biotin was significantly reduced. In fact, the interaction of Avm-W110K and Savm-W120K with biotin was essentially reversible.

In retrospect, it is not particularly surprising that the W→K mutation resulted in the production of a dimeric form of avidin and streptavidin. In both native proteins, the respective tryptophan is contributed to the binding site of one monomer by an extended pair of β-strands from its symmetry-related neighbor, thus comprising a major component of the 'functional' 1→2 (and 3→4) interface of the tetramer [1–4]. Nevertheless, in order to form a dimer from a tetramer, two different interfaces must be impaired, the inference being that both the 1→2 and the relatively weak 1→3 interfaces would be affected by the single, dramatic mutation of tryptophan (110 in avidin and 120 in streptavidin) to lysine. This would result in the formation of stable 'structural' dimers, in which the extensive network of hydrogen-bonding interactions along the 1→4 (and 2→3) interface would presumably be retained. However, this network of hydrogen bonds was sensitive to SDS and its presence caused the dissociation of the dimer into the monomer even at room temperature.

Regarding the observed protease-sensitivity of the avidin mutant, Ellison et al. [30] proposed that the mechanism of biotin-induced protection of native avidin against proteinase K treatment is related to the known closure and rigidification of the sensitive loop between β-strands 3 and 4. In Avm-W110K, biotin failed to protect the mutant against proteolytic attack, thus suggesting that the tertiary structure of the mu-

Table 2
Conserved binding site residues in avidin and streptavidin and their analogues in sea urchin fibropellins

Avidin	Streptavidin	Sea urchin fibropellin[a]
N12	N23	N947
S16	S27	D951[b]
Y33	Y43	Y967
T35	S45	T969
W70	W79	R1002
T77	T90	T1009
F79	W97	W1011
W97	W108	W1028
W110	W120	K1041
N118	D128	D1049

[a]Fibropellin residues were numbered according to GenBank accession number L08692. Analogous residues in the other three fibropellins (L07045, L33861 and L33862) are identical, except that the residue corresponding to tyrosine 33 in avidin is histidine in clones L33862 and L07045.
[b]Unconserved residues in fibropellin are underlined.

tant is altered such that the loop is more accessible to proteolysis.

Mutations of W120 in streptavidin have been described previously. In one study [32], the conservative mutation of this residue to phenylalanine resulted in a reduction of the binding constant for 2-iminobiotin by two orders of magnitude. Its replacement by alanine caused a more severe drop in the affinity of the mutated protein for biotin itself, to a measurable value (1.1×10^{-7} M), reminiscent of the dissociation constant estimated for immobilized monomeric avidin ($K_d = 10^{-7}$ M) [33,34]. Both of these streptavidin W120 mutants were reported to assemble into tetramers.

In a more recent work, Sano et al. [35] described the construction of a mutated form of streptavidin. In this work, histidine 127 was changed to aspartic acid, in order to introduce repulsive forces between subunits 1 and 2 (3 and 4). In addition, the entire 8 residue loop, connecting β-strands 7 and 8, was deleted. W120 was among the residues contained in the deleted loop. The resultant protein was shown to form a soluble dimer in the presence of biotin. The authors assumed that dimeric streptavidin would not be soluble because of the high hydrophobicity of the loop. Since the entire loop was deleted, it is not clear what consequences this would have on the flanking residues that normally form the two separate β-strands in question. Presumably, the residues immediately adjacent to the deleted loop would rearrange into an alternate loop, hence detracting from the lengths of one or both strands. In any case, the authors observed that the stability of the deletion mutant in dimeric form was contingent upon the presence of biotin, the absence of which resulted in the gradual dissociation into inactive monomers.

In contrast, the analogous W→K mutations, described in this communication, were found to be both dimeric and soluble. They also bind biotin reversibly and are stable under long-term storage conditions, even in the absence of biotin. These combined properties may therefore be useful for application in purification studies, in which the recovery of biotinylated proteins or other substances is desired.

Finally, it is intriguing to consider the results of this study in light of the observations that led to the design of the mutants. Specifically, why would sea urchin fibropellins acquire sequences from avidin (or streptavidin)? On the basis of the present study, it would seem that the biotin-binding requirement may not have been the reason, since if high affinity for biotin was needed, then, one would have expected all of the binding residues to have been retained. Indeed, the modification of one of the two homologous tryptophans (W110K and W70R in avidin) caused a significant drop in affinity. Modification of both led to an inactive binding protein.

On the other hand, the stimulus for emulation of the avidin sequence and fold by fibropellins may have been to secure an appropriate structure for facilitating dimerization. In this context, sea urchin fibropellins are extracellular matrix proteins, which may be involved in cell-cell or cell-substratum interactions during embryonic development [7]. The role of the avidin-like domain may be to form dimers and thereby promote protein-protein interactions required for signal transduction. Our results suggest that avidin could serve as an appealing template for such processes, since the dimer afforded by the W110K mutation is a stable one which exhibits a multiplicity of hydrogen bonds across the relevant 1→4 interface. Nevertheless, hydrogen bonds are relatively easier to dissociate than other kinds of bonds, as demonstrated for the W110K mutant dimer, which reverts to the monomer in the presence of SDS. In any case, the exact nature of interaction among fibropellin components, including residual biotin-binding activity, would be dependent on the microenvironment and structure of the native protein. Further evidence in this direction awaits the cloning, expression and analysis of fibropellin components. Preliminary experiments in our laboratory (unpublished results) have indicated that an expressed avidin-like domain of a fibropellin failed to bind to immobilized biotin-containing matrices.

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