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The *AviD*-tag, a NeutrAvidin/avidin specific peptide affinity tag for the immobilization and purification of recombinant proteins

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Abstract

The widespread success of affinity tags throughout the biological sciences has prompted interest in developing new and convenient labeling strategies. Affinity tags are well-established tools for recombinant protein immobilization and purification. More recently these tags have been utilized for selective biological targeting towards multiplexed protein detection in numerous imaging applications as well as for drug-delivery. Recently, we discovered a phage-display selected cyclic peptide motif that was shown to bind selectively to NeutrAvidin and avidin but not to the structurally similar streptavidin. Here, we have exploited this selectivity to develop an affinity tag based on the evolved DRATPY moiety that is orthogonal to known *Strep*-tag technologies. As proof of principle, the divalent *AviD*-tag (*Avidin-Di*-tag) was expressed as a Green Fluorescent Protein variant conjugate and exhibited superior immobilization and elution characteristics to the first generation *Strep*-tag and a monovalent DRATPY GFP-fusion protein analogue. Additionally, we demonstrate the potential for a peptide based orthogonal labeling strategy involving our divalent *AviD*-tag in concert with existing streptavidin-based affinity reagents. We believe the *AviD*-tag and its unique recognition properties will provide researchers with a useful new affinity reagent and tool for a variety of applications in the biological and chemical sciences.

Keywords: Avidin; NeutrAvidin streptavidin; *AviD*-tag; *Strep*-tag; His-tag; Affinity; Orthogonal; Purification; Immobilization

For well over a decade affinity tags have enjoyed widespread use throughout biotechnology and are integral components of numerous research endeavors in the biological sciences [1–4]. These tags have aided tremendously in the production and purification of recombinant proteins [5,6], as well as in the biochemical characterization and functional elucidation of proteins [7,8]. While primarily used for the single-step purification of recombinant proteins from complex mixtures, such as cellular lysates, affinity tags are emerging as useful tools for probing molecular function [8–11], and have recently been used as a convenient means of imaging proteins within live cells [12,13]. Less intrusive than large reporter proteins, fusion peptide bioconjugates can allow for the direct tagging of a protein of interest with a fluorescent indicator such as a quantum dot [14]. However, while fusion peptide based affinity labels provide an efficient means of targeting a protein of interest, specificity is often times sacrificed [15]. Consequently, there is much interest in the development of less invasive and more convenient labeling strategies. Therefore, the development of new peptide based labeling methods that permit the study of proteins in their native state is an attractive goal, not only for the isolation and visualization of proteins under a particular set of conditions, but also for the biochemical classification of many proteins involved in essential cellular processes [16,17].

To date, a wide variety of affinity tags have been developed and are used throughout biotechnology. The most commonly employed affinity tags range from short polypeptide sequences [1,18,19], to whole proteins, which can confer advantageous solubility effects [5]. For example, the specific molecular recognition properties of complete protein domains such as glutathione *S*-transferase and

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the maltose-binding protein have been exploited for recognition of immobilized glutathione and maltose/amylose, respectively [20,21]. In addition to these large whole proteins, small peptide epitopes such as polyhistidine tags [22], which can bind to immobilized metal chelates, as well as the *myc*-tag and *FLAG*-tag [23,24], which can bind to immobilized antibodies, are commonly used for the isolation and immobilization of recombinant proteins. Another small peptide epitope that has gained wide use is the streptavidin specific *Strep*-tag [25]. The development of streptavidin targeted fusion peptides has aided in a variety of unique biochemical applications and has made streptavidin, the non-glycosylated bacterial relative of avidin, the preferred protein in many applications of the (strept)avidin–biotin technologies [26–28]. Having the unfavorable characteristic of reduced specificity due to its high isoelectric point ($pI = 10$) and glycosylated native site [29], avidin is sub-optimal for some biological applications. However, many useful commercial variants of avidin have been recently developed, including the chemically deglycosylated and neutral form of the protein [30], called NeutrAvidin (Pierce). These chemical modifications have reduced non-specific interactions for NeutrAvidin while maintaining its biotin-binding ability [31,32], providing an alternative to streptavidin in many biological applications.

We have recently reported the discovery of a new class of NeutrAvidin/avidin-binding cyclic peptides [33] that may prove to be as useful for a wide variety of applications, as demonstrated for the streptavidin-binding *Strep*-tag [19]. A 6-residue cyclic peptide library, when selected to recognize NeutrAvidin, resulted in the identification of a unique motif: $DX_aAX_bPX_c$ (where X_a = R or L; X_b = S or T; and X_c = Y or W). Several cyclic peptides were individually characterized and shown to bind both NeutrAvidin and avidin with low micromolar dissociation constants, with the peptide DRATPY binding the most tightly with a dissociation constant of 12 μM. It was further shown that this molecular epitope is highly selective for NeutrAvidin/avidin and does not interact with the structurally similar biotin-binding protein, streptavidin. With the aim of developing new reagents for orthogonal labeling in mixed systems, and for providing a new tool for many biological applications, we have developed this new NeutrAvidin/avidin specific motif as an affinity tag. Here, we show that recombinant proteins expressed in conjugation with two copies of this peptide sequence, which we have named the *AviD*-tag (*Avidin-Di*-tag),[1] can be successfully immobilized onto a NeutrAvidin support, thus allowing for the single-step purification of recombinant proteins in yields greater than the original *Strep*-tag [35]. Moreover, the orthogonal nature of the *AviD*-tag and *Strep*-tag may find utility in the multiplexed labeling of distinct proteins in complex biological mixtures.

[1] *Abbreviations used:* *AviD*-tag, *Avidin-Di*-tag; *Avi*-tag, *Avidin*-tag; IPTG, isopropyl-β-D-thiogalactopyranoside; IMAC, immobilized metal affinity chromatography; MALDI, Matrix-Assisted Laser Desorption; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Materials and methods

NeutrAvidin and streptavidin products were obtained from Pierce. All enzymes were purchased from New England Biolabs. All other reagents, unless otherwise noted, were obtained from Sigma.

Molecular cloning

The plasmids for the *Avi*-tag conjugates were constructed by cassette mutagenesis in the pET-Duet vector. The *Avi*-tag cassettes were constructed by extending two overlapping primers with the Klenow fragment of *Escherichia coli* DNA polymerase I. The primers are as follows:

Avi-tag forward: 5′-GATATACCATGGGCTGCGACAG GGCGACGCCGTACTGCGGTGGGAATTCGCTGC AGGG-3′

Avi-tag reverse: 5′-GCATTATGCGGCCGCTTAGTGAT GGTGATGGTGATGCAAGCTTCCCTGCAGCGAA TT-3′

AviD-tag forward: 5′-GCAGGACCATGGGCTGCGATC GCGCGACCCCGTATTGCGGCGGTGGATCCGGC GGTAGCGGCGGTAGTGG-3′

AviD-tag reverse: 5′-TACAGGGAATTCCCACCGCAAT ACGGGGTCGCGCGATCGCAGCCACCGCCGCTA CCGCCACTACCGCCGCT-3′

The *Avi*-tag cassette was cloned into pET-Duet using the NcoI and NotI restriction enzyme sites, and *AviD*-tag was cloned into the resulting plasmid using the NcoI and EcoRI sites. This resulted in two plasmids, each with an N-terminal *Avi*-tag and a C-terminal His-tag. The GFPuv gene was isolated from a plasmid obtained from Clonetech by PCR with the following primers:

GFPuv forward: 5′-GCGGTGGGAATTCGAG TAAAG G-3′

GFPuv reverse: 5′-GTGATGCAAGCTTCCCCCTTTGTA GAGCTCATC-3′

The GFPuv insert was cloned into the *Avi*-tag plasmids between the EcoRI and HindIII restriction enzyme sites using standard protocols to produce pAviGFPuv and pAviDGFPuv. An N-terminal His-tagged fusion of GFPuv that had been previously cloned into pET Duet, pNHTGFPuv, was used as a control construct [36].

The Venus gene was obtained in a plasmid as a generous gift from Dr. Atsushi Miyawaki (RIKEN Brain Science Institute, Japan) and had been previously cloned into pRSF-Duet with an N-terminal His-tag (unpublished results). A C-terminal *Strep*-tag was constructed by cloning into the SalI and NotI restriction sites, to form pStrepVenus, using the following complementary primers:

Strep-tag forward: 5′-CGTACAAGGTCGACGGTGGCG CGTGGCGCCATCCGCAGTTTGGCGGCTAAGCG GCCGCATAATGC-3′
Strep-tag reverse: 5′-GCATTATGCGGCCGCTTAGCCG CCAAACTGCGGATGGCGCCACGCGCCACCGTC GACCTTGTACG-3′

Protein expression and purification

Identical expression and purification strategies were used for each recombinant fusion protein. pAviGFPuv, pAviDGFPuv, pStrepVenus, and pNHTGFPuv were transformed into *E. coli* BL21 (DE3) via electroporation. Single colonies were picked and grown overnight in 100 mL of 2 × YT media with ampicillin at 37 °C with shaking. The overnight culture was used to inoculate 1 L of 2 × YT medium with ampicillin at a starting OD_{600} of 0.08. The cells were grown to an OD_{600} of 0.80 before induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 6 h, the cells were harvested via centrifugation at 4500*g* for 5 min and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Cells were lysed using standard sonication protocols and subsequently centrifuged at 18,000*g* for 40 min at 7 °C. Protein expressed in the soluble fraction was collected. An initial purification was carried out using immobilized metal affinity chromatography (IMAC) in the following manner: the soluble fraction of the cell lysate was incubated with Ni–NTA agarose resin (Qiagen) for 1 h after which it was washed and eluted with increasing concentrations of imidazole (10, 20, 50, and 500 mM). Each protein was further purified by gel filtration chromatography with a HiLoad 16/60 Superdex prep grade column attached to an Amersham FPLC system.

Reflective-phase Matrix-Assisted Laser Desorption (MALDI) mass spectrometry confirmed the fusion proteins' molecular masses to within 3% of the actual molecular weight. Results for each protein are as follows: *Avi*-tag GFPuv: 28,466 (theoretical 29,215); *AviD*-tag GFPuv: 30,996 (theoretical 31,158); N-terminal His-tag GFPuv: 26,719 (theoretical 28,134) and *Strep*-tag Venus: 29,595 (theoretical 30,057). Protein concentration was determined by Trp absorbance at 280 nm following protein denaturation with 6 M guanidine HCl. All subsequent NeutrAvidin related immobilization and chromatographic steps were performed in phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and all subsequent streptavidin related immobilization and chromatographic steps were performed in 100 mM Tris–HCl with 10 mM EDTA, pH 8.0 at 4 °C.

Immobilization of AviD-tag GFPuv on agarose immobilized NeutrAvidin

The GFPuv fusion proteins containing an N-terminal NeutrAvidin/avidin specific peptide affinity tag were incubated with agarose resin containing immobilized NeutrAvidin protein (Pierce) along side the GFPuv control construct. The NeutrAvidin resin was provided as a 6% cross-linked beaded agarose matrix in 50% aqueous slurry with a binding capacity of approximately 50 nmol of biotinylated antibody/mL of immobilized NeutrAvidin protein. For each immobilization assay, approximately 200 pmol of each GFPuv variant was incubated with 100 μL of immobilized NeutrAvidin protein. The mixture was shaken at room temperature for 1 h. Following incubation, each sample was centrifuged at 8000 rpm for 3 min and washed with 100 μL buffer (PBS, pH 7.4). Following each wash, the NeutrAvidin resin for both the tagged and untagged GFPuv variants were exposed to UV light for fluorescence visualization. After five washes, both the tagged and untagged GFPuv NeutrAvidin slurries were incubated with 100 μL biotin (250 μM final) at room temperature for 30 min with shaking. Following treatment with biotin, the resin was washed with 100 μL buffer and exposed to UV light for GFPuv visualization.

Affinity purification using agarose immobilized NeutrAvidin/streptavidin resin

Four hundred microliters of agarose immobilized NeutrAvidin and streptavidin resin (Pierce) was packed into separate disposable polystrene columns and equilibrated with 3 mL buffer. Both immobilized NeutrAvidin and streptavidin resin are described as having binding capacities of approximately 50 nmol of biotinylated target/mL. Approximately 100 μg of FPLC purified tagged GFPuv and Venus were diluted with 2 mL soluble cell lysate and incubated with a gel packed column at room temperature for 1 h with shaking (streptavidin-based products were incubated at 4 °C with shaking). Following collection of the flow-through, each column was washed with 400 μL buffer. After 6 column washes, immobilized protein was eluted with 400 μL of 500 μM biotin. Each eluate was collected in 400 μL fractions. Levels of protein elution and purity were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein bands were visualized by staining with Coomassie brilliant blue. Levels of fluorescence intensity for all chromatographic washes and elutions were measured with a Molecular Devices' SpectraMax Gemini microplate spectrofluorometer.

Results

Immobilization and biotin dependent release of Avi-tag labeled fusion proteins

Recombinant fusion proteins containing the DRATPY moiety were constructed and successfully immobilized onto agarose immobilized NeutrAvidin protein. Two ultraviolet excitable Green Fluorescent Protein (GFPuv) conjugates

encoding two variations of the DRATPY sequence, the *Avidin*-tag (*Avi*-tag) and *Avidin-Di*-tag (*AviD*-tag) were expressed in *E. coli* BL-21 alongside the control GFPuv construct (Fig. 1). To assess immobilization levels for each *Avi*-tag, approximately 6 μg (200 pmol) of each GFP fusion protein was incubated with 100 μL of the NeutrAvidin slurry. Following immobilization, each resin was washed with multiple 100 μL aliquots of buffer and isolated via centrifugation. Following each wash, NeutrAvidin resins incubated with both the tagged and untagged GFPuv variants were photographed under UV light, which enabled direct visualization of GFPuv immobilization (Fig. 2). Following the first wash, fluorescence was seen in both the buffer wash as well as in the NeutrAvidin resin for both the tagged and untagged GFPuv proteins. For the untagged GFPuv variant, minor levels of fluorescence were observed on the NeutrAvidin resin following the second wash and by the third wash, no fluorescence was observed. Fluorescence was observed for the *Avi*-tag-GFPuv fusion protein; however, a stepwise decrease in immobilized protein was witnessed for each subsequent wash. Notably, for the *AviD*-tag-GFPuv fusion protein, uniform fluorescence intensity was observed for each subsequent wash. Following the successful immobilization of the affinity labeled GFPuv fusion protein, 100 μL of a 250 μM biotin containing solution was incubated with each NeutrAvidin resin for 30 min at room temperature with shaking. Upon separation of the buffer and resin, a clearly visible decrease in fluorescence was observed for the NeutrAvidin resin immobilized with *AviD*-tag-GFPuv accompanied by the corresponding appearance of fluorescence in the elution buffer wash (Fig. 2).

Purification of fusion proteins from cell lysate

We next sought to develop a general strategy for the one-step purification of recombinant proteins under gentle conditions. We explored the practicality and applicability of our affinity tag with gravity-flow purification from cell lysate. For each purification exercise, approximately 100 μg of *Avi*-tag and *AviD*-tag labeled GFPuv was mixed with 2 mL of prepared *E. coli* lysate. Each crude cell lysate mixture containing affinity labeled GFPuv protein was then subjected to gravity-flow purification. Following flow-through collection, 400 μL aliquots of buffer were used to wash the column. After six washes, the NeutrAvidin resin was treated with 500 μM biotin and collected in 400 μL fractions. The wash and elution samples were subsequently analyzed by 15% SDS–PAGE. The results confirmed our earlier observations that the designed divalent peptide sequence, *AviD*-tag, is the more potent affinity tag. SDS–PAGE analysis suggests the lower affinity of the monovalent *Avi*-tag resulted in higher levels of protein elution during the washing procedure (Fig. 3a), while the divalent *AviD*-tag was able to withstand rigorous washing and could be successfully eluted upon addition of biotin (Fig. 3b). Further, *AviD*-tag labeled protein was shown to be largely homogenous, containing no major contaminants.

Demonstration of orthogonality and applicability in comparison to the Strep-tag

Members of the motif $DX_aAX_bPX_c$ (where X_a = R or L; X_b = S or T; and X_c = Y or W) have been shown to not

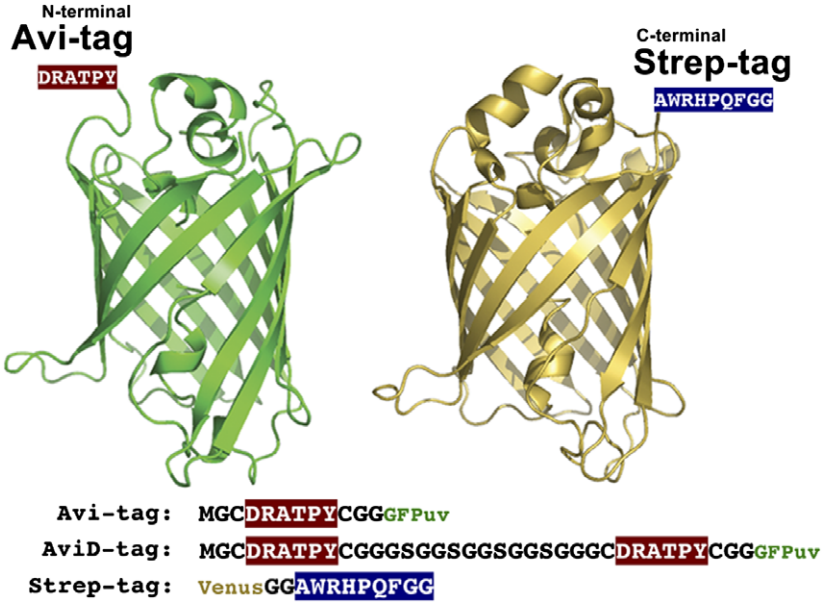

Fig. 1. Ribbon representations of GFPuv and Venus fusion proteins. The NeutrAvidin/avidin specific monovalent *Avi*-tag and divalent *AviD*-tag (sequences shown in red) were expressed in conjugation with GFPuv (PDB ID: 2EMD) on its N-terminal domain. The streptavidin specific *Strep*-tag (sequence shown in blue) was expressed in conjugation with the Yellow Fluorescent Protein, Venus (PDB ID: 1MYW), on its C-terminal domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

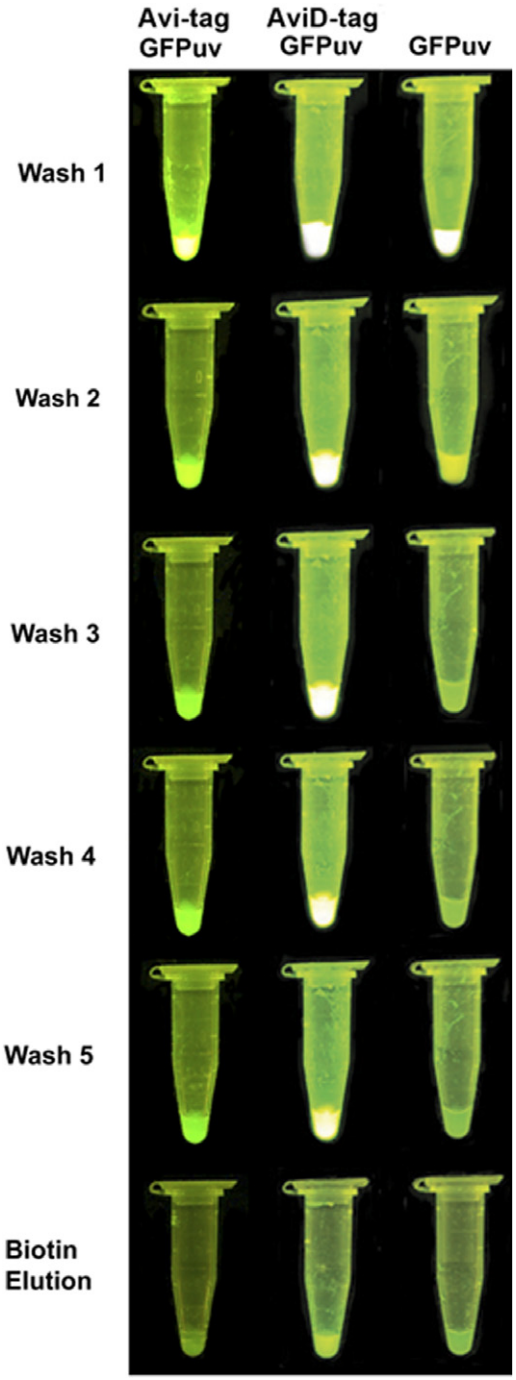

Fig. 2. Immobilization of *Avi*-tag GFPuv and *AviD*-tag GFPuv fusion proteins on agarose immobilized NeutrAvidin resin. Two hundred picomoles of tagged GFPuv and untagged GFPuv were incubated with 100 μL of NeutrAvidin resin for 1 h at room temperature. Following incubation, NeutrAvidin resins were centrifuged, washed with 100 μL buffer and photographed under UV light for direct visualization of GFPuv fluorescence. Following the fifth wash, NeutrAvidin resins were incubated with 100 μL of a 250 μM biotin solution for 30 min at room temperature followed by UV visualization.

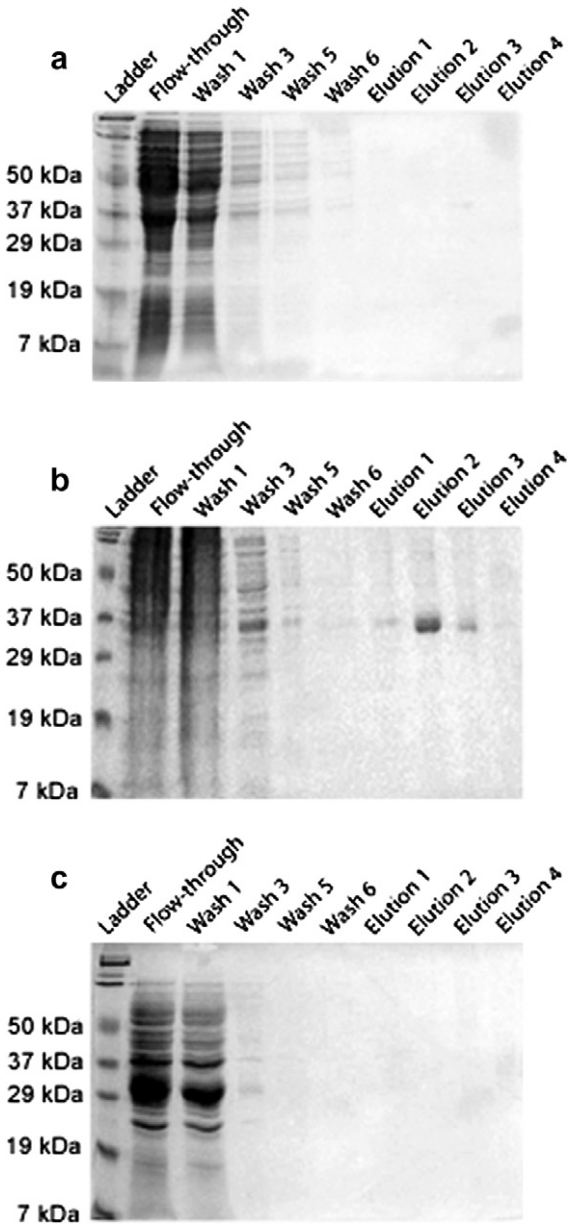

Fig. 3. Affinity purification of fusion proteins from cell lysate. (a) Four hundred microliters of agarose immobilized NeutrAvidin incubated with cell lysate containing 100 μg of *Avi*-tag GFPuv. (b) Four hundred microliters of agarose immobilized NeutrAvidin incubated with cell lysate containing 100 μg of *AviD*-tag. (c) Four hundred microliters of agarose immobilized streptavidin resin incubated with cell lysate containing 100 μg of *AviD*-tag GFPuv. (a–c) Following 6 column washes, immobilized protein was eluted with 500 μM biotin. Levels of protein elution and purity were resolved on a 15% SDS–polyacrylamide gel under reducing conditions.

bind streptavidin [33,34], if applied to fusion peptide technologies, peptide discrimination amongst receptors could become a powerful tool for the study of many biological systems. To explore the specificity of *AviD*-tag for NeutrAvidin, the GFPuv fusion protein was subjected to gravity-flow purification against agarose immobilized streptavidin resin under conditions identical to those of the NeutrAvidin resin. Subsequent SDS–PAGE analysis of buffer washes and biotin elutions revealed the fusion protein was unable to bind to the column, eluting off entirely by the second wash (Fig. 3c). To further demonstrate the potential of an orthogonal peptide based labeling system,

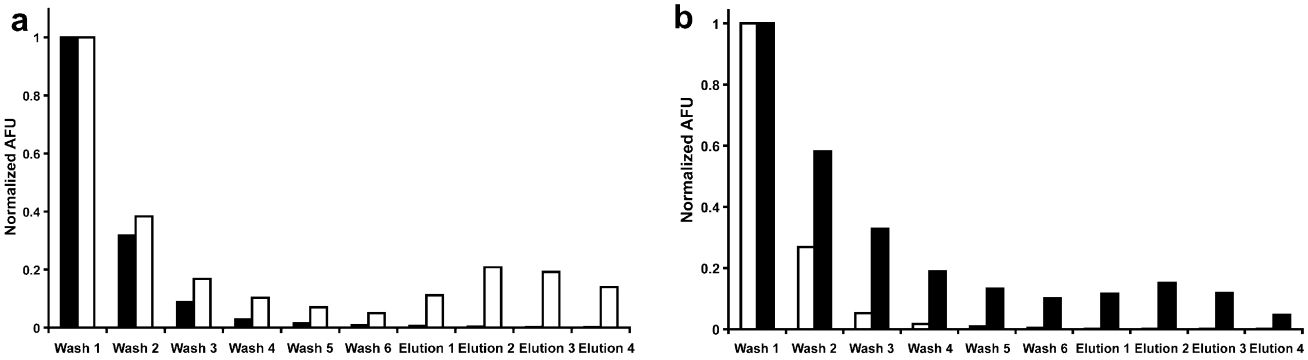

Fig. 4. Orthogonal binding of the *AviD*-tag and *Strep*-tag. Three nanomoles of *Avi*-tag-GFPuv (a) and *Strep*-tag Venus (b) were incubated with 400 μL agarose immobilized NeutrAvidin (white) or streptavidin (black) resin. After 1 h, each column was washed successively with six buffer washes. Immobilized protein was eluted with 500 μM biotin and collected in 400 μL fractions. Fluorescence for each column wash and eluate was measured with a Molecular Devices' SpectraMax Gemini microplate spectrofluorometer. Fluorescence intensity is normalized to Wash 1 for each fusion protein.

a Yellow Fluorescent Protein variant (Venus) containing a streptavidin specific C-terminal *Strep*-tag was constructed (Fig. 1). The Venus fusion protein was produced under conditions identical to those previously described. Following purification, approximately 3 nmol of recombinant protein was mixed with cell lysate. The crude cellular mixture was incubated separately with 400 μL of both immobilized NeutrAvidin and streptavidin resin at 4 °C. While the *Strep*-tag fusion protein was successfully immobilized and purified from the streptavidin solid support, it was unable to bind to immobilized NeutrAvidin. In addition to the *AviD*-tag's orthogonal nature, fusion proteins labeled with the divalent construct were purified in yields greater than that of *Strep*-tag labeled Venus (Fig. 4a), which was observed to have significantly higher levels of protein dissociate from off the resin during wash procedures.

Discussion

Throughout the biological sciences, there is continued interest in developing fast and convenient fusion peptide technologies. To date, these short molecular epitopes have aided tremendously in numerous biochemical studies and promise to facilitate the functional elucidation of uncharacterized gene products [4,8]. Yet, while a wide variety of affinity tags have been developed and characterized throughout the years, drawbacks associated with each molecular recognition epitope calls for the continued development of new orthogonal handles. In some cases there are elution problems associated with the extremely high affinity of antibody derived affinity tags, which in some cases been cleverly circumvented through the use of non-denaturing antibody epitopes such as Softags [37,38], while affinity tags derived from antibodies such as the *myc*-tag or the *Flag*-tag traditionally require denaturing conditions for antibody dissociation [23,24]. Further, whole protein-tags such as glutathione *S*-transferase or the maltose-binding protein often require treatment with site-specific proteases for subsequent biochemical characterization of the desired protein [18]. Even affinity tags that have gained widespread use throughout the life sciences can have potential drawbacks such as the His-tag, which can associate with a host of metal chelating contaminants [35]. Therefore, the development of new molecular recognition technologies for the isolation, purification, and characterization of proteins is of continuing utility. To this end, we have developed the *AviD*-tag, a NeutrAvidin/avidin specific fusion peptide affinity tag capable of protein immobilization, and purification. Moreover, the orthogonal nature of the *AviD*-tag and *Strep*-tag may find numerous applications in cellular labeling technologies [39].

Our previous efforts towards the characterization of the phage-display selected motif $DX_aAX_bPX_c$ (where $X_a = R$ or L; $X_b = S$ or T; and $X_c = Y$ or W) showed that a high level of selectivity exists for NeutrAvidin/avidin versus streptavidin [33]. The observed >1000-fold selectivity, in parallel with the necessity for molecular recognition technologies for NeutrAvidin/avidin, immediately suggested the utility of this cyclic peptide motif for protein purification and immobilization. Consequently, recombinant fusion proteins containing the DRATPY moiety were constructed. We had previously shown that the linear DRATPY moiety exhibits significantly reduced and nearly negligible affinity for its cognate receptors (<80-fold), and therefore worked under the assumption that if the disulfides were unformed neither the *Avi*-tag nor the *AviD*-tag would show appreciable affinity for NeutrAvidin.

Using the ultraviolet excitable GFPuv as our model protein, we produced two fusion proteins encoding the DRATPY peptide: a monovalent variant (*Avi*-tag) expected to exhibit faster off-rates and lower elution profiles, and a divalent variant (*AviD*-tag) expected to produce higher levels of protein immobilization and purification as a result of its lower dissociation constant (Fig. 1). We successfully immobilized and eluted the *AviD*-tag labeled GFPuv onto NeutrAvidin immobilized agarose resin (Fig. 2), in yields greater than *Avi*-tag labeled GFPuv. To further confirm the necessity of the divalent design and the formation of the disulfide bridge, gravity-flow purification against immobilized NeutrAvidin resin from cell lysate

was performed with both fusion proteins. Significantly higher levels of protein elution were clearly observed with the divalent affinity tag following affinity purification and subsequent SDS–PAGE analysis (Fig. 3b).

As previously described, members of this phage-display selected motif had been shown to be 1000-fold more selective for NeutrAvidin/avidin versus streptavidin. To demonstrate that the orthogonal behavior of the phage-display selected peptides had successfully translated to the divalent fusion peptide, *AviD*-tag labeled GFPuv was subjected to gravity-flow purification against streptavidin immobilized agarose resin. As expected, the divalent *AviD*-tag labeled fusion protein was not immobilized by the streptavidin support (Fig. 3c). To further assess the orthogonal nature of the system, a Venus (Yellow Fluorescent Protein variant of GFP) fusion protein containing a C-terminal streptavidin specific *Strep*-tag, a previously well-characterized affinity tag that binds streptavidin, with a reported dissociation constant of 37 μM, was constructed [26]. This fusion protein was subjected to gravity-flow purification procedures identical to those described for each NeutrAvidin specific fusion protein. Surprisingly, the *AviD*-tag proved to be more effective than the *Strep*-tag. Immobilized *AviD*-tag was shown to be retained at higher levels than that of the *Strep*-tag and allow for significantly higher levels of protein elution following addition of biotin (Fig. 4). Consequently, the *AviD*-tag's ability to effectively immobilize proteins under physiological conditions suggests that it will be of value in a host of protein purification and immobilization exercises.

Additionally, the commercial availability of many (strept)avidin conjugates suggests the *AviD*-tag's utility in a myriad of unique biological applications. While already established as useful tools in protein purification, immobilization, and characterization, genetically fused affinity peptide tags can be utilized for selective biological targeting [13,40]. Expressed in conjugation with known receptors, specific fusion peptide motifs can aid in a host of unique cell-surface molecular recognition applications and provide tremendous support for the elucidation of many biochemical phenomena. For example, utilization of peptide motifs that provide orthogonal binding to streptavidin and avidin conjugated to organic fluorophores or quantum dots can provide new and exciting methods for protein detection, imaging and multiplexing. In summary, the orthogonal recognition properties of our *AviD*-tag will possibly provide researchers in the biological sciences with a valuable new tool capable of a wide variety of unique cellular applications.

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