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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: