The highly dynamic oligomeric structure of bradavidin II is unique among avidin proteins

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Abstract: Bradavidin II is a biotin-binding protein from *Bradyrhizobium japonicum* that resembles chicken avidin and bacterial streptavidin. A biophysical characterization was carried out using dynamic light scattering, native mass spectrometry, differential scanning calorimetry, and isothermal titration calorimetry combined with structural characterization using X-ray crystallography. These observations revealed that bradavidin II differs from canonical homotetrameric avidin protein family members in its quaternary structure. In contrast with the other avidins, bradavidin II appears to have a dynamic (transient) oligomeric state in solution. It is monomeric at low protein concentrations but forms higher oligomeric assemblies at higher concentrations. The crystal structure of bradavidin II revealed an important role for Phe42 in shielding the bound ligand from surrounding water molecules, thus functionally replacing the L7,8 loop essential for tight ligand binding in avidin and streptavidin. This bradavidin II characterization opens new avenues for oligomerization-independent biotin-binding protein development.

Keywords: ligand binding; oligomeric state; dynamic structure; structural cooperativity

Abbreviations: AVR2, avidin-related protein 2; AVR4, avidin-related protein 4; DSC, differential scanning calorimetry; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; FT-ICR, Fourier transform ion cyclotron resonance; ITC, isothermal titration calorimetry; L1,2, loop between beta strands 1 and 2; MS, mass spectrometry; RMSD, root-mean-square deviation; T_m , transition midpoint of thermal denaturation

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Introduction

Avidin and streptavidin are the workhorses of the life sciences. These biotin-binding proteins are used in numerous applications and their structures and functions have been widely studied (for review, see Refs. 1 and 2). Avidin was originally isolated from chicken egg white, whereas streptavidin is a structurally and functionally analogous protein that was isolated from the bacterium *Streptomyces avidinii*.³ Recent studies have revealed there are several other avidins that functionally resemble (strept)avidin. Of these proteins, bradavidin,⁴ rhizavidin,⁵

shwanavidin,⁶ xenavidin,⁷ tamavidin 2,⁸ and chicken avidin-related proteins 2 (AVR2)⁹ and 4 (AVR4)¹⁰ have been structurally characterized, and the majority share similarities with (strept)avidin and consist of eight-strand beta barrels assembled into tetramers.

Avidins are characterized as beta-barrel proteins with a high affinity to biotin (with a K_d in the pM to fM range). Their high ligand-binding affinity is based on a perfect structural complementarity between the protein and biotin ligand (also known as vitamin H), high number of hydrogen bonds, and ligand-binding site architecture. Avidins are relatively thermostable, and some are also resistant to proteases and harsh chemical conditions, so they are widely used in life science applications. All avidins characterized to date, with the exceptions of rhizavidin¹¹ and shwanavidin,⁶ use residues from two adjacent subunits in ligand recognition. Therefore, although biotin binding to avidin is noncooperative in nature,¹² ligand binding and the protein's oligomeric state are interdependent properties in avidins. Tetrameric avidins may be not optimal as fusion partners or labeling purposes, because oligomerization of the fusion partner may change its biological function and oligomerization in general leads to avidity effects. In addition, oligomerization increases molecular weight of the fusion protein. Therefore, in many applications, monomeric avidins could be beneficial. The oligomericity of (strept)avidin has been genetically manipulated by applying mutations to its subunit interfaces (for example, see Refs. ¹³ and ¹⁴). The outcomes of these studies have typically produced proteins with relatively low biotin affinities, and the engineered proteins have also been found to be less thermostable than wild-type proteins. A promising recent study has shown that monomeric streptavidin with relatively high affinity to biotin and increased thermostability can be obtained via genetic engineering.¹⁵ Importantly, nature appears to offer good alternatives to avidin and streptavidin in relation to oligomeric states, with naturally dimeric rhizavidin and shwanavidin as good examples.

This study describes the three-dimensional (3D) structure and biophysical analysis of bradavidin II, which is a biotin-binding protein derived from *Bradyrhizobium japonicum* that resembles other avidins in its primary sequence (Fig. 1). Bradavidin I and II are both from the genome of *B. japonicum*. They share only low sequence identity (34% as calculated by NCBI blastp), whereas majority of the residues associated with ligand binding are conserved in both of the proteins. Bradavidin I has been found to structurally resemble canonical avidin members.⁴ Now, for the first time, we report a highly dynamic oligomeric state for an avidin family member. Biophysical analyses suggest that bradavidin II is monomeric at low protein concentrations but forms

transient oligomers (mainly dimers and trimers) at higher protein concentrations. Structural X-ray crystallography analysis revealed the assembly of bradavidin II subunits to be a loosely packed tetramer in which the canonical interactions between subunits of tetrameric avidin family members (chicken avidin, streptavidin, bradavidin, etc.) are missing, and the relative orientation between the subunits is unique. The bradavidin II structural information presented here sheds new light on tight ligand binding and may enable the design of more sophisticated biotin binders with better control over protein oligomeric states.

Results

Protein production

Initial attempts to crystallize bradavidin II by following Helppolainen *et al.*¹⁶ were unsuccessful. All resulting crystals showed poor diffraction. To increase protein yield and quality, novel protein batches were produced with a fermentor by fed-batch protocol as described.¹⁷ Following 2-iminobiotin purification, two protein forms were detected by SDS-PAGE analysis. To further identify these protein forms, the protein sample was analyzed using high-resolution Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS). We detected 12.5 and 16.1 kDa protein forms, with the latter representing protein accompanied by extra residues from the expression vector and resolved by MS/MS experiments (for details, see Supporting Information Fig. S1). To resolve the problem described above, a second stop codon was added to the expression vector and homogenous 12.5-kDa protein was obtained after 2-iminobiotin affinity chromatography purification with yield of more than 10 mg pure protein per liter of bacterial culture.

The structure of bradavidin II

Bradavidin II was crystallized and solved in three forms (two apo and one biotin complex) (Table I). All three bradavidin II forms displayed the typical tertiary structure of the avidin family, which consists of eight antiparallel β -strands forming a β -barrel. The asymmetric units of all crystal forms contained one (apo Form-A, resolution 1.9 Å), two (apo Form-B, resolution 1.7 Å), or four (biotin complex, resolution 1.75 Å) monomers, none of which resembled any common avidin canonical dimer or tetramer arrangements, even during symmetry operations, suggesting the presence of a monomer in a crystalline state. In practice, structural alignment of bradavidin II structures with tetrameric avidins such as chicken avidin (PDB 2AVI and 1VYO) or streptavidin (PDB 1STP) or dimeric avidins such as rhizavidin (PDB 3EW2) and shwanavidin (PDB 3SZJ) clearly show that the interface contacts between subunits are not present

Figure 1. Sequence alignment. The bradavidin II sequence is aligned with the following previously characterized avidins: AVD, chicken avidin; Str, streptavidin from *Streptomyces avidinii*; RhizAv, rhizavidin from *Rhizobium etli*; SwAv, shwanavidin from *Shewanella denitrificans*; BradI, bradavidin I from *Bradorhizobium japonicum*; BradII, bradavidin II. The residues in close contact with biotin are highlighted in gray and bolded. Cysteine residues corresponding to disulfides close to the biotin-binding site are shaded with black. The Phe residues unique to bradavidin II and shwanavidin are shaded with light blue. The first residue within the resolved 3D structure of bradavidin II is shaded with green. Arg17 appears functionally important as discussed in Figure 2 and is therefore shaded red. The secondary structure elements are shown according to bradavidin II. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in bradavidin II. Bradavidin II is a relatively short protein consisting of only 115 amino acids [this number includes three N-terminal residues (QTV) from the ompA signal peptide used in protein expression¹⁸], making it the smallest known member of the avidin family. Some strand-connecting loops are relatively short (Fig. 1), and although in most avidins there is only a single β -turn to connect strands $\beta 1$ and $\beta 2$, in bradavidin II there are five β -turns (L1,2, L4,5, L5,6, L6,7, and L7,8) among the seven loops. In addition, L5,6 exhibits a unique short helical segment with Cys-69 in the disulfide bridge to connect with L3,4. The L3,4 loop, which is critical for biotin binding, has the same size as most avidins. In this context, the L3,4 in bradavidin II maintains remarkable sequence and conformational similarity with that of shwanavidin.⁶ Like the dimeric avidins, bradavidin II lacks a Trp residue from L7,8, which is critical for the protein's high affinity to biotin and oligomeric integrity in tetrameric avidins,^{19,20} also demonstrated earlier by mutagenesis.^{13,21} A

disulfide bridge connecting Cys-44 and Cys-69 to L3,4 and L5,6, respectively, is available in bradavidin II, rhizavidin,⁵ and shwanavidin.⁶ In addition, bradavidin II has a phenylalanine in its L3,4 loop (Phe-42) at an identical position to the phenylalanine of shwanavidin, which was shown to compensate for the lack of Trp from L7,8⁶ (Fig. 2, see also Fig. 1).

The biotin-binding site

The biotin-binding site in bradavidin II shares many features with other dimeric and tetrameric avidins, which consist of conserved hydrophobic and polar residues (Fig. 1). The L3,4 loop of most avidin apo forms exhibits flexibility or open conformations. Two molecules are available in the asymmetric unit of crystal Form-B, in which L3,4 adopts two distinct conformations [Fig. 3(A)]. The open conformation leaves the biotin-binding site completely exposed to solvent, whereas the binding site in the closed form is completely sealed. The segment in L3,4 loop N-

Table I. Data Collection and Refinement Statistics

Bradavidin II	Apo Form-A	Apo Form-B	Biotin complex
ESRF beamline	ID 14-4	ID29	ID23-1
Wavelength (Å)	0.93	0.98	0.97
Space group	C2	P1	C2
Unit cell parameters (Å)	$a = 62.1,$ $b = 46.1,$ $c = 47.2, \beta = 116.7^\circ$	$a = 34.8, b = 46.1,$ $c = 46.4, \alpha = 106.6^\circ,$ $\beta = 106.9^\circ, \gamma = 106.8^\circ$	$a = 119.1, b = 95.4,$ $c = 49.9, \beta = 113.6^\circ$
Resolution range (Å) (outer resolution shell)	42.17–1.9 (1.93–1.9)	40.66–1.7 (1.73–1.70)	71.8–1.75 (1.78–1.75)
Unique reflections	9133	23,010	51,302
Redundancy	3.1	2.2	3.5
$R_{\text{sym}} (I)^a$	6.1 (56.0)	8.3 (27.2)	7.1 (75.4)
Completeness	96.4 (87.3)	86.0 (83.6)	99.8 (99.9)
I/σ	22.1 (2.0)	16.0 (2.4)	20.0 (1.76)
Number of protein atoms	829	1662	3420
Number of ligand atoms	—	—	64
Number of solvent atoms	27	135	404
R -factor	0.236	0.211	0.19
R -free ^b	0.272	0.256	0.235
B -factor			
Protein	33.96	26.01	21.7
Biotin	—	—	18.4
Solvent	44.26	37.66	37.6
RMSD			
Bond length	0.014	0.014	0.016
Bond angle	1.71	1.69	1.75
Ramachandran			
Favored	94.2	90.4	89.4
Allowed	5.8	9.6	10.6
Generously allowed	0.0	0.0	0.0
Disallowed	0.0	0.0	0.0

^a $R_{\text{sym}}(I) = \sum |I - \langle I \rangle| / \sum I$.

^b Test set consists of 5% for all data.

terminal to the disulfide bridge (residues 38–43) displays different conformations, whereas the remainder of the loop exhibits an almost identical conformation in the resolved structures (Fig. 3). Thus, it can be concluded that L3,4 is flexible in solution and its crystal structures have discrete open and closed loops. The L3,4 loop exhibits a closed conformation in apo Form-A like that of Form-B.

In the biotin complex [Fig. 3(B)], the ligand forms a network of polar interactions with bradavidin II as in all other avidins. The main difference is a lack of conserved Tyr from the $\beta 3$, which was replaced by Phe-35 in bradavidin II (Fig. 4). Previous studies have shown that this residue contributes to the biotin-binding affinity of chicken avidin²² and streptavidin,²³ but is not essential for high affinity. The L3,4 loop adopts a closed conformation, as in all four asymmetric unit monomers, and forms two conserved H-bond interactions, one with the biotin ureido nitrogens and one with the carboxylate oxygens (Fig. 4). In addition to polar interactions, biotin resides in a hydrophobic box formed by four aromatic residues including Trp-75, Trp-90, Phe-66, and Phe-42. Moreover, the disulfide bridge also interacts with biotin, which phenomenon is known to be present in rhizavidin and shwanavidin.^{5,6} A unique

feature that was not observed in prior avidin studies is the presence of Arg-17 in L1,2 and its contribution to the biotin-binding site (Fig. 2). The side chain of Arg-17 extends toward the biotin molecule and forms a polar interaction with Asp-106 (the latter forms an H-bond interaction with biotin, see Fig. 4). In addition, the side chain of Arg-17 also seals the biotin-binding site from the side of the bicyclic ring (Fig. 2).

Bradavidin II shows a relatively high affinity to biotin

Previous studies revealed that bradavidin II tightly binds to biotin and has a dissociation rate constant comparable with those of other avidins, such as rhizavidin¹¹ and bradavidin,²⁴ but significantly higher than those measured for avidin and streptavidin.¹⁶ However, the biotin-binding affinity was not quantitatively determined. As in a previous study,¹⁶ we found the direct determination of biotin-binding affinity to be impossible with isothermal titration calorimetry (ITC), because the affinity exceeded the upper limit of the method. Therefore, we used competitive ITC²⁵ to more precisely determine the affinity by first injecting desthiobiotin into the sample cell, followed by biotin injection. This analysis was performed with a range of competing desthiobiotin

Figure 2. Biotin availability in the binding site. Surface presentation of the biotin-complexed bradavidin II (upper left), bradavidin II Δ R-17 (modeled R*, upper right), streptavidin (bottom, left), and shwanavidin (bottom, right). In streptavidin, the Trp-120 (shown in orange) from an adjacent monomer caps the biotin molecule in its binding pocket, almost completely leaving the carboxylate partially available to solvent. In bradavidin II and shwanavidin, the Trp is not available because of the oligomeric state and is compensated by a Phe (Phe-43 in purple and Phe-42 in dark cyan for shwanavidin and bradavidin II, respectively), thereby sealing biotin in its binding site. In bradavidin II, Arg-17 from the L1,2 β -turn provides an additional element in biotin solvent unavailability. Removing R-17 (*in silico*) now termed Δ R-17 results in a binding site that is more available to solvent, similar to that as in shwanavidin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ligand concentrations. The competitive ITC revealed a $K_d = 1.2 \pm 0.2 \times 10^{-7} M$ for the bradavidin II–desthiobiotin complex, and the displacement of $500 \mu M$ desthiobiotin by biotin revealed a $K_d = 6.6 \pm 1.5 \times 10^{-11} M$ for the bradavidin II–biotin complex (Table II; Fig. 5). The K_d and ΔH values over the range of desthiobiotin concentrations are shown in Supporting Information Table S1.

Bradavidin II has lower thermal stability relative to other avidins

The thermal stability of bradavidin II was studied by using a differential scanning calorimeter (DSC). Protein samples were analyzed by varying the protein concentration and pH. Unfortunately, the experiments at pH 7 were not successful, most likely

because the protein aggregated (see below for further analyses). In contrast, the experiments at pH 3 and pH 5 were more successful, although extended storage at pH 5 led to protein precipitation. Precipitated proteins were observed already after 1 week storage at pH 5 buffer at $4^\circ C$ with the naked eye. Dimethyl sulfoxide (DMSO) (1%) was found to slower the precipitation. However, precipitated proteins were observed after 2 weeks storage also in the presence of 1% DMSO at pH 5. Partially reversible unfolding was observed at a pH of 3 in the presence of biotin (Fig. 6). The addition of $3\times$ excess molar biotin significantly stabilized the protein, which is an indication of high ligand-binding affinity as well as a characteristic of avidins¹ (Table III). We found no significant differences in the transition midpoints of thermal

Figure 3. The biotin-binding site of apo (top) and biotin-complexed (bottom) bradavidin II. For the apo bradavidin II the L3,4 loop adopts two distinct conformations as observed in the Form-B crystals. These open (magenta) and closed (cyan) conformations depict the flexibility of the L3,4 loop. The L3,4 loop in the biotin complex form adopts a closed conformation similar to the conformation observed in the apo form. A Phe-42 contributed by the L3,4 forms hydrophobic interactions with biotin, resulting in almost complete ligand unavailability to the solvent. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

denaturation (T_m) along the range of protein concentrations in the absence of the ligand. In contrast, T_m was found to be slightly dependent on the protein concentration in the presence of the ligand (Table III).

The oligomeric state of bradavidin II is dependent on protein concentration and pH

To study the oligomeric state of bradavidin II, the hydrodynamic radii of bradavidin II samples were analyzed by dynamic light scattering (DLS), and molecular weight estimates were calculated with a globular protein standard curve from the DLS instrument manufacturer (Malvern Instruments, Worcestershire, UK). In addition to determining the hydrodynamic radius at a fixed temperature, we also studied the behavior of the protein sample during heating with a temperature range of 20–90°C.

Bradavidin II oligomeric state was found to be concentration dependent. A monomer-oligomer transition was observed at low pH (0.5M acetic acid, pH 3) when the protein concentration was varied from 0.2 to 3.8 mg/mL [Fig. 7(C)]. Representative size distribution graphs are shown in Supporting Information Figure S2. At a low concentration (0.4 mg/mL), the protein was mostly monomeric and heat denaturation did not induce protein aggregation (Supporting Information Table S2). Oligomers were detected at a higher protein concentration (1 mg/mL), and protein aggregated at increasing temperatures with a transition temperature at 50°C. However, biotin stabilized the protein and affected its behavior after thermal unfolding; no aggregation was observed at 90°C even at a protein concentration of 1 mg/mL (Supporting Information Table S2). This finding suggests that thermal unfolding leads to aggregation at a lower temperature range, but biotin makes it possible to bypass this range, and thermal denaturation at high temperatures (DSC experiments, Table III) yields soluble denatured molecules that can refold upon cooling with the help of biotin (Fig. 6).

The oligomeric state of bradavidin II was also found to be dependent on pH. Oligomeric species were detected at room temperature and a pH of 5 (50 mM sodium phosphate and 100 mM NaCl), and when the temperature was increased [Fig. 7(B)]. As expected, the presence of biotin stabilized the

Figure 4. Schematic representation of the hydrogen-bond interactions of bradavidin II with biotin. In principle, biotin forms the canonical network of H-bond interactions as observed in other known avidins. In this context, the biotin carboxylate forms two interactions (one oxygen each) with Ala-43 N and Ser-71 O γ . The biotin ureido ring exhibits a cardinal difference that was not previously observed. The conserved Tyr residue in all other avidins (from strand β 3) is replaced by Phe-35 in bradavidin II, so the conserved avidin H-bond interaction with ureido oxygen is lacking. The remaining interactions with the bicyclic ring system remain identical.

Figure 5. Isothermal titration calorimetry analysis for ligand binding. (A) Bradavidin II was titrated with desthiobiotin, which revealed a moderate affinity with a $K_d = 1.2 \pm 0.2 \times 10^{-7} M$. (B) To determine binding affinity for bradavidin II–biotin complex, biotin was titrated into a sample containing a mixture of desthiobiotin (500 μM) and bradavidin II (5 μM). This analysis revealed a high-affinity interaction of $K_d = 6.6 \pm 1.5 \times 10^{-11} M$. The original data are shown in the upper panels and the integrated heats for each injection in the lower panel are shown together with the fit.

protein, and the onset of aggregation was detected at higher temperatures when compared with samples devoid of biotin (Table III). However, biotin could not prevent temperature-induced protein aggregation at pH 5, as seen at a pH of 3. We observed increased thermal stability of bradavidin when pH was raised from pH 3 to pH 5 (Table III). This is not surprising, because pH 3 could be considered already quite harsh condition. Further increment in pH did not cause further stabilization of the protein. This may be caused by two different reasons: first, bradavidin II appears to form loose oligomers at neutral pH. It is possible that these oligomers accelerate protein unfolding at elevated temperature, thus causing decrease in the thermal stability. Second, it is possible that the protein has optimal thermal stability at mildly acidic pH.

DLS experiments performed at pH 7 (50 mM sodium phosphate and 100 mM NaCl) revealed mostly aggregated species [Fig. 7(A)] with protein concentrations of 0.4 or 1.0 mg/mL, which was not surprising, because the precipitation was visually observable. Therefore, we carried out additional experiments at a pH of 7 with a lower protein concentration (0.25 mg/mL), which also revealed large protein assemblies, the majority of which were dissolved with a slight increase in temperature. Aggregation was observed after heating the sample further, and biotin was found to increase the transition temperature (Supporting Information Table S2) as in the pH 3 and pH 5 treatments. Overall, the poor solubility of bradavidin II at pH 7 makes it challenging to characterize its oligomeric behavior in this condition.

Table II. Isothermal Titration Calorimetry Analysis for Biotin and Desthiobiotin Binding to Bradavidin II

Protein	Ligand	$K_a \pm SD$ ($\times 10^9 M^{-1}$)	$K_d \pm SD$ ($\times 10^{-9} M$)	$\Delta H \pm SD$ (kcal/mol)	ΔS (cal/mol/K)
Bradavidin II	Desthiobiotin	0.008 ± 0.001	123.5 ± 14.6	-21.7 ± 0.3	-37.8
Bradavidin II	Biotin	N.D. ^a	N.D.	-26.5 ± 0.4	N.D.
Bradavidin II–desthiobiotin	Biotin (competitive)	15.2 ± 0.4	0.066 ± 0.015	-23.1 ± 0.1	-27.1

The analysis was carried out at a pH of 3 at 40°C.

^a The binding constant could not be determined because of a high binding affinity exceeding the capability of the instrument.

Figure 6. Thermal stability of bradavidin II. Differential scanning calorimetry scans of bradavidin II (0.4 mg/mL) in 0.5M acetic acid (pH 3) and in ammonium acetate (pH 6.8) containing 0.1% DMSO. The first heating scan (dark gray line) and rescan (gray line) of bradavidin II are shown both in the absence of biotin (A, C) and in the presence of biotin (B, D).

We also used native MS to characterize the oligomericity of bradavidin II as a function of protein concentration (Fig. 8). Native MS is a powerful tool for studying protein quaternary structure in solution because different oligomeric states are directly separated and quantified. All experiments were performed in 25 mM ammonium acetate (pH 6.8), and the protein concentration was varied from 0.25 to 1 mg/mL. Bradavidin II was mainly monomeric at a concentration of 0.25 mg/mL, with a small amount of dimeric protein. At higher concentrations, increasing amounts of higher oligomeric forms (mainly dimers and trimers) were observed. The protein was mostly trimeric at a concentration of 1 mg/mL, but other oligomers (up to a tetramer) were also observed. These results are consistent with the DLS data acquired at pH 3. No protein aggregation was observed in the 25 mM ammonium acetate buffer pH 6.8 used for native MS up to the highest concentration (1 mg/mL) as determined by visual inspection. The biotin efficiently complexed with bradavidin II with expected stoichiometries, indicating a high binding affinity. However, biotin did not

cause significant changes in oligomeric distribution (data not shown). In accordance with these findings, DLS experiments performed in 25 mM ammonium acetate buffer pH 6.8 mainly revealed proteins with hydrodynamic radii of 2.24–2.66 nm (with estimated molecular weights of 22.3–33.3 kDa) within a protein concentration range of 0.2–0.6 mg/mL [see Fig. 7(A) corresponding to 0.4 mg/mL]. No radical changes in the average particle size were observed (hydrodynamic radius 2.38–3.19 nm) after 1 week of storage at +4°C, which indicates that the proteins are more soluble in this buffering system than in sodium phosphate buffer. DSC analysis revealed similar transition midpoint temperatures for thermal unfolding at pHs of 5 and 3, and thermal unfolding was found to be irreversible both in the absence and presence of biotin (Fig. 6).

Bradavidin II oligomeric state was also assessed by using SDS-PAGE method described by Bayer *et al.*²⁶ Only monomeric forms of bradavidin II were observed in both of the temperatures (20 and 80°C) studied and addition of biotin had no effect on the oligomeric state (Supporting Information Fig. S3).

Table III. The Unfolding Temperatures (T_m) of Bradavidin II in the Absence and Presence of Threefold Molar Excess of Biotin Analyzed by Differential Scanning Calorimetry

Condition used	T_m ($^{\circ}\text{C}$)	
	– Biotin	+ Biotin
pH 3, 0.2 mg/mL	50.9 $\pm$ 1.4	83.9 $\pm$ 0.7
pH 3, 0.4 mg/mL	50.3 $\pm$ 1.1	84.6 $\pm$ 0.8
pH 3, 0.6 mg/mL	50.3 $\pm$ 0.9	85.2 $\pm$ 1.3
pH 5, 0.2 mg/mL	66.5	90.4
pH 5, 0.4 mg/mL	66.3	93.2
pH 5, 0.6 mg/mL	66.5	85.7 ^a
pH 6.8, 0.2 mg/mL	62.4	88.4
pH 6.8, 0.4 mg/mL	63.3	90.0
pH 6.8, 0.6 mg/mL	63.7	90.9

^a A weak second peak corresponding to apo form was observed ($T_m = 67.2^{\circ}\text{C}$), most likely indicating the presence of protein forms not accessible to biotin (aggregates).

Comparative analysis was carried out for chicken avidin variant, which appeared as monomer only when heated to 80°C in the absence of biotin, as expected. Chicken avidin tetramers were not visible in the Coomassie-stained gel, and therefore we silver stained the gel (Supporting Information Fig. S3b). The silver-stained gel further confirmed the absence of any oligomeric species of bradavidin II. Essentially, the same results were obtained without chemical acetylation of bradavidin II, but the sample treated at 20°C in the presence of biotin showed more diffuse band (data not shown), most probably because of high isoelectric point (theoretical pI 9.34).

Discussion

Bradavidin II resembles other avidins in terms of primary structure and shares certain special sequence characteristics with rhizavidin, shwanavidin, and bradavidin (Fig. 1). Therefore, we were interested in structurally characterizing this protein. Although the 3D structure revealed the tetrameric assembly of the asymmetric unit in the presence of biotin, the assembly differs from the tetramers of other avidins and lacks the majority of key subunit interactions conserved in other avidin protein family members, suggesting a monomeric or transient oligomeric structure for bradavidin II. In agreement with this structural hypothesis, biophysical characterization revealed relatively low thermal stability and a highly dynamic oligomeric state, which was found to be strongly dependent on the environment.

Tight biotin–avidin binding has been widely observed, and high ligand-binding affinity is thought to be closely associated with avidin oligomerization. In terms of protein structure, oligomeric assembly not only refers to interactions between ligand and protein subunits but also contributes to the protein function, for example, by shielding the ligand

from surrounding water. In practice, a deuterium-hydrogen exchange between chicken avidin and the surrounding solution radically decreases when biotin is bound, indicating global communication between the avidin and its ligand.²⁷ It has also been observed that ligand binding significantly thermally stabilizes the oligomeric structure of avidin family members.

Figure 7. Oligomeric state of bradavidin II under different conditions. The molecular weight estimates of bradavidin II were analyzed by DLS. Bradavidin II was analyzed in the absence (–) and presence (+) of biotin in 0.5M acetic acid containing 0.1% DMSO (pH 3), in 50 mM sodium phosphate buffer containing 100 mM NaCl and 0.1% DMSO (pH 5), in 25 mM ammonium acetate containing 0.1% DMSO (pH 6.8), and in 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 7). (A) The oligomeric state of bradavidin II (0.4 mg/mL) was analyzed at different conditions. (B) The oligomeric state of bradavidin II (0.4 mg/mL) at pH 5 varies at different temperatures. (C) At pH 3, the oligomeric state of bradavidin II increases at increasing protein concentration, both in the absence and presence of biotin. M indicates monomer (12.24 kDa), D indicates dimer (24.48 kDa), Tr indicates trimer (36.72 kDa), and Te indicates tetramer (48.96 kDa).

Figure 8. FTICR-MS analysis of bradaavidin II. Native MS experiments reveal heterogenic distribution of oligomeric species, the higher order oligomers being more abundant at high protein concentrations. Experiments performed under denaturing conditions show purely a monomeric protein species with a molecular weight of 12569.42 Da, which is close to the expected molecular weight of Bradavidin II of 12569.42 Da (see also Supporting Information Fig. S1). Different peaks were assigned to oligomeric species and labeled in the figure as follows: M = monomer, D = dimer, Tr = Trimer, and Te = tetramer. The charge state of each species is shown.

From this perspective, it is surprising that there are avidins capable of tight biotin binding without canonical oligomericity (tetrameric quaternary assembly). The first examples of these proteins were rhizavidin and shwanavidin, which exhibited dimeric quaternary structures. This bradaavidin II study appears to be the first discovery of a natural avidin without a clearly defined oligomeric state, and the monomers are therefore functionally independent. We found it challenging to biophysically characterize bradaavidin II because of its atypical behavior in several assays. For instance, we were not able to determine the protein's characteristics by gel filtration chromatography, and the poor solubility of the protein at neutral and basic pH in phosphate-based buffers also complicated its analysis.

A primary concern was to find what makes bradaavidin II capable of tightly binding with biotin. First, the hydrogen bond network between the

protein and its ligand (Fig. 4) was quite well conserved when compared with other avidins (Fig. 1), the only exception being the tyrosine residue in all other avidin loop L3,4s (Tyr-33 in chicken avidin), which is replaced by phenylalanine in bradaavidin II. Second, the protein has a disulfide bridge, judged by MS and X-ray analyses, to stabilize the L3,4, which is a highly mobile loop in several other avidins. Disulfide apparently decreases mobility, and therefore makes it possible to more efficiently shield the bound ligand. Interestingly, rhizavidin, shwanavidin, and bradaavidin I share this structural moiety with bradaavidin II (Fig. 1). Third, bradaavidin II appears to have a unique mechanism to compensate for the absence of the well-conserved Trp residue, which contributes to the biotin binding from the adjacent subunit in chicken avidin (Trp-110), streptavidin (Trp-120), and in other tetrameric avidins. In addition to phenylalanine 42, which is also found in

shwanavidin,⁶ bradavidin II contains arginine 17, which helps to bury biotin in the surrounding solvent.

Ligand-binding analysis by ITC revealed exothermic binding both in the case of biotin and desthiobiotin. Both analyses had an entropic contribution against binding, but the effect was more pronounced in the case of desthiobiotin (Table II). This finding could be explained by the less rigid structure of desthiobiotin in solution relative to biotin,

although both of them were virtually immobile when bound to protein. When compared with chicken avidin's extremely tight binding to biotin ($K_d = 10^{-15}$ – $10^{-16}M$),^{28,29} the biotin-binding affinity of bradavidin II decreased by more than 1000-fold. Previous studies indicated that the dissociation rate constant measured by displacing radioactive biotin associated with bradavidin II is significantly higher than that of chicken avidin.¹⁶ Thus, it is clear that a more rapid dissociation rate provides an explanation for the decrease in bradavidin II biotin-binding affinity relative to avidin and streptavidin. An earlier study revealed binding affinities of $1 \times 10^{-12}M$ and $5 \times 10^{-13}M$ for avidin–desthiobiotin interaction at pHs of 4 and 7, respectively.²⁸ Therefore, the ligand-binding affinity decrease of bradavidin II in desthiobiotin relative to avidin (~100,000-fold) appears to be larger than that of biotin.

The oligomeric state of bradavidin II was found to be highly dependent on solution conditions (pH, temperature, and ionic composition). When exposed to neutral pH in sodium phosphate buffer, the protein started to form large complexes and precipitated (Fig. 7). When the pH was decreased to 5, the protein stayed in solution, but it aggregated during extended storage. Bradavidin II showed good solubility at a pH of 3. DLS experiments performed at pH 3 (0.5M acetic acid) indicated that the protein hydrodynamic radius increased with increasing protein concentrations, suggesting concentration-dependent oligomerization (Fig. 7). Native MS experiments performed in 25 mM ammonium acetate (pH 6.9) indicated increasing amounts of protein dimers, trimers, and tetramers in solution with an increasing protein concentration (Fig. 8). These findings are in agreement with the crystal structures, which indicated loose protein packing into tetrameric assemblies and ultimately did not resemble canonical avidin

Figure 9.

Figure 9. Structural comparison of bradavidin II to chicken avidin. (A) Cartoon presentation of chicken avidin tetramer PDB 1VYO³³ with certain key residues in its 1-2/1-3 and 1-4 interfaces highlighted as VdW spheres. (B) Comparison of bradavidin II to avidin 1-2/1-3 interface. Bradavidin II structure superimposed with one of the subunits of avidin (gray subunit 1). Avidin Val115 residues are shown as spheres and bradavidin II Lys103 as sticks. This analysis highlights the incompetence of bradavidin II to maintain the canonical 1-2/1-3 subunit interface structure seen in chicken avidin. (C) Comparison to avidin 1-4 interface. Bradavidin II residues Phe72, Tyr61, and Arg74 are shown in stick representation and labeled. Three hydrophobic avidin residues from two different subunits are shown as follows: white, Thr76 from subunit 1; light gray, Gly65 from subunit 1; gray, Val78 from subunit 1; light blue, Thr76 from subunit 4; blue, Gly65 from subunit 4; and dark blue, Val78 from subunit 4. Again, bradavidin II appears not to have conserved residues at these positions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tetramers. The absence of oligomers was also judged by SDS-PAGE-based method (Supporting Information Fig. S3). The behavior of bradavidin is unique within avidin family, but other proteins have shown examples of concentration- and pH-dependent oligomerization. Arylsulfatase has been shown to form dimers in neutral and basic conditions but converts to octameric state in the mildly acidic environment.³⁰ Similar to bradavidin 2, this protein was found challenging to study using size-exclusion chromatography. LptA is a periplasmic lipopolysaccharide transport protein from *Escherichia coli*. This protein was studied using SEC-LS method, which indicated concentration-dependent oligomerization in a concentration range of 0–80 μM studied.³¹ Unfortunately, we have not found explanation for the discrepancy between the gel filtration analysis results obtained by Helppolainen *et al.* and those observed in this study. It is possible that the 58.4-kDa form observed by Helppolainen *et al.* is somehow associated with the leaky stop codon.

We found the thermal unfolding characteristics of bradavidin II to be atypical among the avidins. To our knowledge, all avidins characterized so far experience irreversible thermal unfolding. Bradavidin II instead demonstrated a largely reversible thermal unfolding at a pH of 3 in the presence of biotin (Fig. 6). We believe that this phenomenon is associated with the oligomerization properties of bradavidin II. Protein refolding is a much simpler reaction if the protein has no oligomeric structure or only weak interactions between subunits. Successful oligomeric protein refolding might require assistance in shielding the hydrophobic protein–protein interfaces to cause rapid aggregation, whereas monomers can unfold and refold independently. SDS has been known to prevent streptavidin aggregation after thermal unfolding,³² which supports this model.

Another important question is why bradavidin II does not form tetramers or dimers such as those of other avidin family members. We used the previously determined X-ray structure of chicken avidin (1VYO)³³ and positioned the bradavidin II structure by using avidin as a template (Fig. 9). A clear sign of a potentially important obstacle relative to the canonical avidin assembly was noted in the residue corresponding to Val115 in chicken avidin [Fig. 9(A,B)]. This residue forms extensive hydrophobic contacts in the center of the avidin tetramer and the corresponding residue in bradavidin II is a lysine 103, which is clearly incompatible with the role of valine. A lysine residue was also found at this position in shwanavidin, which is reportedly a dimer.⁶ Finally, rhizavidin, which is also a dimeric avidin-like protein, has a glutamine at this site (Ref. ¹¹, see also Fig. 1). Moreover, a closer look into the 1-4 dimer interface [Fig. 9(A,C)] explains why bradavidin II does not form a stable dimer, unlike

rhizavidin and shwanavidin. Hydrophobic contacts are formed by a row of tiny residues of Thr76, Gly65, and Val78 in the chicken avidin 1-4 dimer. In bradavidin II, these residues are replaced by Phe72, Tyr61, and Arg74, which would cause clashes in an avidin-like pairing between subunits.

In conclusion, bradavidin II has an atypical 3D structure for a member of the avidin protein family, with no clear signs of oligomeric assembly into a well-defined quaternary structure. This finding was supported by biophysical analyses, which showed oligomeric state variations to be dependent on the protein concentration and solution conditions. DSC data showed that the protein has quite efficient refolding capacity after heat-induced unfolding at low pH. Although the ligand-binding affinity determined for bradavidin II ($K_d = 6.6 \pm 1.5 \times 10^{-11}\text{M}$) was not as high as in well-known streptavidin and chicken avidin, bradavidin II can be characterized as an avidin family member. The structural properties of bradavidin II may assist protein engineers in creating modified biotin-binding proteins with characteristics suitable for life science applications. Because of its weak oligomerization tendency, bradavidin II could be a better candidate for developing fusion partners for large proteins than chicken avidin or streptavidin.

Materials and Methods

Protein production and purification

Bradavidin II was expressed with a pET101/D expression plasmid. The original signal peptide of the protein was replaced with an ompA secretion signal as described in previous works.^{16,18} A second stop codon was added by standard molecular biology methods to the expression construct used in the previous study¹⁶ to ensure an end product of the correct size. Bradavidin II protein was produced in *E. coli* BL21-AI cells in a 7.5-L fermentor (Labfors Infors 3, Infors AG, Bottmingen, Switzerland) using the fed-batch mode as previously described,¹⁷ and the resulting cell mass was collected by centrifugation and lysed with a homogenizator (Avestin Emulsiflex-C3, Avestin, Ottawa, ON, Canada). The lysate was clarified by centrifugation and subjected to 2-iminobiotin affinity chromatography (Affiland, Liège, Belgium). Protein was eluted with 0.5M acetic acid and verified as pure and homogenous by SDS-PAGE and MS.

Bradavidin II crystallization, data collection, and structure solution

Apo bradavidin II crystals were obtained by microbatch method at 20°C. A 1- μL drop contained equal amounts of 3.9 mg/mL protein in 0.5M acetic acid (pH 3) mixed with 30% PEG 4000, 0.2M MgCl_2 , and 0.1M Tris-HCl (pH 8.5). Cube-shaped crystals appeared within a week and reached a final size of 0.05 mm within 2 weeks. A different batch with rod-

shaped crystals was obtained by combining 3.4 mg/mL protein with 0.12M magnesium formate. Diffraction parameters were improved later by the addition of 0.1M NaBr. A bradavidin II–biotin complex was obtained by incubating the protein with 5 μ L of saturated *d*-biotin solution (1.5 mg biotin in 1 mL of 0.5M acetic acid) for 0.5 h at 4°C. Crystals from this complex were obtained by microbatch method at 20°C. A 1- μ L drop contained equal amounts of 3.9 mg/mL protein–biotin complex in 0.5M acetic acid (pH 3) mixed with 10% PEG 20K, 2% 1,4 dioxane, and 0.1M bicine (pH 9). Hexagon-shaped crystals appeared within 1 month. Another crystallization condition was applied by mixing the bradavidin II–biotin complex in a reservoir containing 20% PEG 3350 and 0.1M sodium citrate (pH 3.5). Crystals appeared within 5 days and reached their final sizes within 14 days. Before freezing, apo crystal forms were briefly suspended in a cryoprotectant solution containing 25% glycerol and crystallization solution. Biotin complex crystals were suspended in crystallization solution containing 25% ethylene glycol.

Crystallographic data for apo bradavidin II Form-A were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France at beamline ID14-4. The data were collected from a single crystal at 100 K using an Oxford Cryosystem Cryostream cooling device (as were all data described below) on an ADSC Q315 CCD detector. The crystal belonged to monoclinic space group C2 with cell parameters $a = 62.1$ Å, $b = 46.01$ Å, $c = 47.2$ Å, and $\beta = 116.7^\circ$ (Table I). All data were indexed, integrated, and scaled using the HKL2000 suite.³⁴

The Form-A structure was solved by molecular replacement using PHASER^{35,36} and implemented in the CCP4 suite. Search models included the monomer from a Swiss-Model^{37,38} bradavidin II homology model derived from the structure of rhizavidin (PDB code 3EW1 with 47% sequence similarity).⁵ Matthew's coefficient indicated the presence of one bradavidin II molecule in the asymmetric unit ($V_M = 2.32$ Å³/Da, 47% solvent content). The resulting PHASER solution at a resolution range of 40.0–3.5 Å gave a monomer in the asymmetric unit. The structure was initially refined using the rigid body protocol in REFMAC,³⁹ resulting in an *R*-value of 48.7% and *R*-free of 49.6%. The structure was further refined with REFMAC5 via the restrained refinement mode at a resolution range of 42.17–1.9 Å, and solvent molecules were added with ARP/wARP.⁴⁰ After several iterative cycles of refinement and model building with Coot,⁴¹ the *R*-value and *R*-free reached final values of 23.6 and 27.2%, respectively (Table I). The final model contained 827 protein atoms and 27 solvent molecules (Table I).

Additional data were collected for the apo bradavidin II displaying different symmetry (designated at Form-B) at the ESRF ID-29 beamline on a PILATUS

6M pixel detector at 100 K. Crystals from the triclinic P1 space group with cell parameters $a = 34.77$ Å, $b = 46.12$ Å, $c = 46.45$ Å, $\alpha = 106.59^\circ$, $\beta = 106.92^\circ$, and $\gamma = 106.82^\circ$ exhibited somewhat better resolution (1.7 Å) than the Form-A (Table I). The structure of the Form-B triclinic bradavidin II was solved by PHASER using the bradavidin II refined Form-A monomer as the search model at a resolution range of 40–3.5 Å. The solution indicated the presence of two bradavidin II monomers in the asymmetric unit with a V_M value of 2.83 Å³/Da and 56.6% solvent. The structure was initially refined in a similar protocol for the Form-A apo bradavidin II. The rigid body refinement resulted in an *R*-value of 31.9% and *R*-free of 33.6% at a resolution range of 40–3.5 Å. The structure was further refined at a resolution range of 40–1.7 Å, resulting in a final *R*-value of 21.1% and *R*-free of 25.5% after the addition of water molecules using ARP/wARP. The final model contained 1662 protein atoms and 135 solvent molecules (Table I).

Crystallographic data for the bradavidin II–biotin complex data were collected at ESRF ID-29 on an ADSC Q315 CCD detector. The crystals belonged to monoclinic space group C2 with cell parameters $a = 119.01$ Å, $b = 95.43$ Å, $c = 49.95$ Å, and $\beta = 113.6^\circ$ (Table I). The structure of the biotin complex was solved by PHASER (50–4 Å) using the bradavidin II refined monomer as the search model. The solution indicated the presence of four bradavidin II monomers in an asymmetric unit ($V_M = 2.96$ Å³/Da, 58.4% solvent). The structure was further refined at a resolution range of 71.8–1.75 Å, resulting in a final *R*-value of 19.2% and *R*-free of 23.7% (Table I). Electron density maps clearly indicated the presence of biotin molecules in the corresponding binding sites, which were fitted with Coot.⁴¹ The final model contained 3429 protein atoms, 404 solvent atoms, and 64 biotin atoms (Table I). The structures were deposited in the RCSB-PDB database with the following access codes: Bradavidin II apo Form-A, 4GGR; Bradavidin II apo Form-B, 4GGT; and Bradavidin II biotin-complex, 4GGZ.

Biophysical characterization

A MicroCal VP-Capillary DSC (GE Healthcare Life Sciences, Microcal, Northampton, MA) was used to determine the unfolding temperature (T_m) of bradavidin II. Samples were analyzed in 0.5M acetic acid (pH 3) and in 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 5). It was not possible to perform the analysis in 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 7) because of sample aggregation difficulties. However, we were able to perform DSC analysis using a 25 mM ammonium acetate buffer at pH 6.8. The measurements were conducted in the absence and presence of biotin using a 3:1 molar ratio of biotin per bradavidin II subunit. A scanning rate of 2°C/min was used

and the experiment was performed by running a temperature scan from 20 to 130°C. A small amount of DMSO (0.1%) was included to make the biotin soluble at a low pH. The data were analyzed using Microcal Origin 7.0 software.

The protein hydrodynamic radius was determined by DLS with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Six parallel measurements were carried out for each sample (100 µL) at 25°C. The molecular weight of the protein was estimated from the hydrodynamic radius using a globular protein standard curve from the manufacturer. A molecular weight of 12.24 kDa was used as the M_w of the monomer for determining the protein oligomeric state.

All mass spectrometric experiments were performed with a 12-T APEX-Qe FT-ICR instrument (Bruker Daltonics, Billerica, MA) equipped with an Apollo-II electrospray ion source. All protein samples were buffer-exchanged with 10 mM ammonium acetate (pH 6.8) before analysis. The samples were further diluted with acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v) for denaturing MS or 25 mM ammonium acetate (pH 6.9) for native MS experiments. All ion source parameters (temperature and voltage settings) were optimized to maintain weak protein–protein interactions and all other instrument parameters were adjusted to maximize ion transmission at m/z 1500–4000. The instrument was operated and the data were processed and analyzed with Bruker XMASS 7.0.8 software. To calculate the neutral (most abundant isotopic or average) masses for each oligomeric protein form, mass spectra were charge-deconvoluted with a dedicated Tcl script.

A DLS thermal scanning experiment was performed by gradually increasing the sample temperature with a Peltier element embedded within the Zetasizer Nano ZS instrument. These experiments were carried out in a quartz cuvette (Hellma 104-QS, Hellma GmbH & Co. KG, Müllheim, Germany) in 300-µL samples. The temperature was raised from 20 to 90°C in 5°C steps, and the sample was incubated for 5 min at each temperature before measurement. The resulting data were analyzed by plotting the molecular weight estimate and deriving the count rate over the temperature.

ITC was performed with a Microcal VP-ITC (GE Healthcare, Life Sciences, Microcal, Northampton, MA). The measurements were completed in 0.5M acetic acid (pH 3) at 40°C. Samples were degassed before analysis by stirring for 5 min at 39.5°C in a MicroCal ThermoVac. First, 5 µM bradavidin II was titrated with 15-µL aliquots of 75 µM desthiobiotin solution, and the result was analyzed by using the “one sites of binding” method in Microcal Origin 7.0. A competitive ITC was performed for a mixture of 5 µM bradavidin II in the presence of excess desthiobiotin (15, 75, 150, 200, 300, and 500 µM) and 75 µM biotin was injected

in 15-µL aliquots into the mixture of bradavidin II and desthiobiotin. The resulting data were analyzed using the “competitive binding” method included in the data analysis software package provided by the manufacturer. This method, displacement ITC, is based on the protocol described by Sigurskjold and it allows to determine the binding isotherm of a ligand that is competitively inhibited in its binding to a protein with identical and independent binding sites. The injection reaction heats were integrated using Microcal Origin 7.0 and nonlinear least-squares fitting was used to obtain K_a , ΔH , and n .²⁵

The oligomeric state of bradavidin II and chicken avidin variant with lowered isoelectric point (Niederhauser *et al.*, unpublished) was studied by using thermal treatment in the presence of SDS, but in the absence of reducing agent using the method described earlier.²⁶ Before analysis, the proteins were chemically acetylated to decrease their isoelectric point using sulfo-NHS acetate (Pierce). The protein samples were treated for 20 min in SDS in the presence or absence of biotin. Then, the oligomeric state was analyzed using SDS-PAGE stained using Coomassie blue or with silver. PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used as molecular weight standard.

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