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Bradavidin II from *Bradyrhizobium japonicum*: A new avidin-like biotin-binding protein

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ABSTRACT

A gene encoding an avidin-like protein was discovered in the genome of *B. japonicum*. The gene was cloned to an expression vector and a protein, named bradavidin II, was produced in *E. coli*. Bradavidin II has an identity of 20–30% and a similarity of 30–40% with previously discovered bradavidin and other avidin-like proteins. It has biochemical characteristics close to those of avidin and streptavidin and binds biotin tightly. In contrast to other tetrameric avidin-like proteins studied to date, bradavidin II has no tryptophan analogous to the W110 in avidin (W120 in streptavidin), thought to be one of the most essential residues for tight biotin-binding. Homology modeling suggests that a proline residue may function analogously to tryptophan in this particular position. Structural elements of bradavidin II such as an interface residue pattern or biotin contact residues could be used as such or transferred to engineered avidin forms to improve or create new tools for biotechnological applications.

1. Introduction

Avidin, the chicken (*Gallus gallus*) egg-white protein and its bacterial (*Streptomyces avidinii*) analogue streptavidin, are biotin-binding proteins which have exceptionally high affinity ($K_d \sim 10^{-14} - 10^{-16}$ M) for the vitamin D-biotin (referred to here as biotin). Both proteins are tetrameric, consisting of four identical subunits, numbered 1 to 4 according to Livnah et al. [1]. Each subunit is made up of eight antiparallel β-strands with the overall shape of a β-barrel, and each subunit binds one molecule of biotin. The tight biotin-binding capacity of these proteins has been widely exploited in a plethora of applications in life sciences and technology across a range of biochemical, pharmaceutical and biophysical applications [2]. A great number of genetically modified forms of avidin and streptavidin, collectively called (strept)avidin, have been generated during the past 15 years [3,4].

The gene for the new bacterial avidin, named bradavidin, was discovered in *Bradyrhizobium japonicum* [5], which is a nitrogen-fixing and root nodule-forming symbiotic bacterium on the soybean. Soon after bradavidin was discovered, the genome of *B. japonicum* was observed to contain a putative gene for another avidin-like protein, which was named bradavidin II. Bradavidin genes may thus form a gene family similar to those of avidin and avidin-related genes (AVRs) in the chicken [6]. However, the biotin-binding proteins from *B. japonicum* have low similarity (meaning amino acids having similar characteristics), the identity (meaning conserved amino acids) between the amino acid sequences of bradavidin and bradavidin II being approximately 24% and the similarity 36%. For comparison, bradavidin II and chicken avidin have an identity of 27% and a similarity of 38%, those with streptavidin being 19% and 32% (Supplementary material).

One of the most important amino acid residues responsible for the tight biotin interaction in avidin is W110 and in streptavidin W120 [1,7]. Both reach from the neighboring subunit and form a lid-like structure on the top of the biotin-binding pocket, strengthening the binding affinity [1]. Subunits 1 and 2 (also 3 and 4) thus form a so-called functional pair. Subunits 1 and 4 (2 and 3) form a structural pair by merit of several mutual hydrogen bonds and hydrophobic interactions between subunits. All the avidin-like biotin-binding proteins studied to date are tetrameric and possess a tryptophan residue analogous to W110 in avidin, except for one, rhizavidin, isolated from the bacterium *Rhizobium etli* [8]. Rhizavidin is a dimer and has no tryptophan in the corresponding position. Similar to rhizavidin, bradavidin II possesses no tryptophan analogous to the W110 in avidin but has a tetrameric structure (Figs. 1 and 2). In this respect its quaternary structure assembly and mode of biotin-binding clearly warrant closer study.

Bradavidin II was characterized by studying its primary structure, quaternary structure assembly, ligand-binding capacity, thermal stability, protease sensitivity and immunological cross-reactivity. Based

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on its (strept)avidin-like characteristics with conserved amino acids and tight biotin-binding affinity in relation to other avidin-like proteins, bradavidin II was taken to belong to the avidin protein family.

2. Material and methods

2.1. Construction of the expression vectors

Genomic DNA of *B. japonicum* (DDBJ AP005955.1) was used as a template for bradavidin II gene amplification by PCR. An original signal sequence of bradavidin II was replaced with a bacterial secretion signal, OmpA from *Bordetella avium* [9]. The gene was extended using the stepwise elongation method. The first forward primer for the 5′ end was 5′-GCCGCCGTTACGGCCTCTGGTGTTGCCTCGGCTCAGACCGTGGAGGCCATGGCG-CAGGGG-3′. The PCR product was extracted from a 1.5% agarose gel and subjected to the second PCR with the following primer for the 5′ end, 5′-CACCATGAACAAACCCTC-CAAATTCGCTCTGGCGCTTGCCTTCGCCGCCGTTACGGCCTC-3′, including CACC at its 5′ end, necessary for directional TOPO® cloning (Invitrogen, Carlsbad, CA, USA). The reverse primer for the 3′ end was in both cases 5′-CTACCATACGCGCGTGAATAT-3′. The amplified DNA was extracted from the agarose gel and cloned to the expression vector pET101/D by TOPO® cloning protocol (Invitrogen). The fidelity of the constructed expression vectors was always verified by DNA sequencing with a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.2. Production and purification of recombinant bradavidin II

The recombinant expression vector was transformed into *E. coli* BL21-(AI) cells. The transformants were cultured in LB medium supplemented with 0.1% (w/v) glucose and 100 μg/ml ampicillin (Sigma/Aldrich, St. Louis, MO, USA) at 26 °C with rotation at 225 rev/min. Culturing was maintained until OD_{600} 0.2–0.3 was reached, followed by addition of L-arabinose to a final concentration of 0.2% (w/v). Cultivation was continued at 26 °C for approximately 20 h, thereafter the cells were collected by centrifugation (5000 *g*, 4 °C, 10 min). The cell lysate was sonicated three times for 2 min (60% duty cycle, 5 s on, 2 s off) using Vibra Cell™ (Sonics & Materials Inc. Danbury, CT, USA) [9], and the protein was isolated by affinity chromatography on a 2-iminobiotin (Affiland, Liège, Belgium) column and eluted with 0.5 M ethanoic (acetic) acid [10]. The protein was analyzed in an SDS-PAGE gel (15%) with subsequent Coomassie Brilliant Blue staining. A theoretical extinction coefficient (ε) of 33,570 M^{-1} cm^{-1} at 280 nm and a theoretical molecular mass (12,568 Da) were determined using the PepStats program from the European Molecular Biology Open Software Suite (EMBOSS) according to the sequence corresponding to mass spectrometry analysis result. Concentration of the protein was determined spectrophotometrically measuring absorbance at 280 nm. The theoretical extinction coefficient and the theoretical molecular mass were used to calculate the protein concentration from an equation, $A=\varepsilon cl$, where A is an absorbance, ε a theoretical extinction coefficient, c concentration and l path-length (1 cm).

2.3. Primary structure analysis

The Needle program (EMBOSS) was used to create pairwise sequence alignments. A multiple sequence alignment of the avidin-like proteins was generated using the Clustal W program.

2.4. Phylogeny tree

An unrooted phylogeny tree was obtained by the Neighbor-joining method with *p*-distance matrix and conducted in MEGA4.0 [11]. The confidence levels were obtained using the bootstrap method (1000 replicates) [12].

2.5. Homology modeling

Homology models of bradavidin II were made using Modeller 9v2 [13]. Avidin (PDB 2AVI) or streptavidin (PDB 1MK5) were used as templates and the pairwise sequence alignments done using Clustal W. The cysteine pair in bradavidin II was forced to form a disulphide bridge.

2.6. Structural analysis

Mass spectrometry was performed on a 4.7-T APEX-Qe Fourier-transform ion cyclotron resonance (FT-ICR) instrument (Bruker Daltonics), interfaced to an external electrospray ionization (ESI) source (Apollo-II™) [8]. The protein sample in ammonium acetate buffer (10 mM, pH 6.9) was diluted to ~5 μM with CH_3CN/H_2O/HOAc (49.5:49.5:1.0, v/v) and directly infused at a flow rate of 1.5 μl/min. ESI-generated ions were accumulated in the hexapole ion trap for 1.0 s and transferred to the Infinity ICR cell for trapping, excitation and detection. A total of 128 co-added (1-Mword) time-domain transients were fast-Fourier-transformed prior to magnitude calculation and external frequency-to-m/z calibration with respect to the ions of an ES Tuning Mix (Agilent Technologies). The data were processed with the use of Bruker XMASS 7.0.8 software.

The purified bradavidin II was analyzed by SDS-PAGE (15% gel) with subsequent Coomassie Brilliant Blue staining. Protein samples (50 μM in ethanoic acid) were prepared by adding an equal volume of SDS-sample buffer in the presence and absence of the reducing agent, β-mercaptoethanol (β-ME), boiled in a water bath for 15 min and chilled on ice.

The quality and size of the purified protein were analyzed by HPLC gel filtration in the absence and presence of biotin. The protein samples (0.1 mg/ml) were analyzed in a SuperDex 200 10/300 GL column with a phosphate buffer (50 mM Na-phosphate, 650 mM NaCl) in the liquid phase. The column was calibrated with a gel filtration standard (thyroglobulin, gamma-globulin, ovalbumin (bovine), myoglobin, and vitamin B12; Bio-Rad Laboratories Inc., CA, USA) [5]. To reduce putative intersubunit disulfide bridges, bradavidin II was incubated in the presence of (10% v/v) DTT (dithiothreitol) prior to gel filtration at 37 °C for 1 h.

2.7. Heat and protease stability

The sensitivity of bradavidin II to proteinase K was analyzed in the absence and presence of an excess of biotin which was 3 mol of biotin per biotin-binding site [14]. The final concentration of bradavidin II was 16 μM (phosphate buffer). The samples were incubated at 37 °C for 5, 30, 60 min, 3 and 22 h in the presence of proteinase K (1:25 w/w). Immediately after incubation an equal volume of SDS-PAGE sample buffer was added and the samples were boiled in a water bath for 15 min and frozen. All samples were analyzed simultaneously in SDS-PAGE gel (15%) and stained with Coomassie Brilliant Blue.

Differential scanning calorimetry (DSC) was used to determine the heat-induced unfolding temperatures (T_m) of bradavidin II, as previously described [8,15,16]. The samples were dialyzed against phosphate buffer (50 mM Na-phosphate, 100 mM NaCl, 1 mM β-mercaptoethanol, pH 7), lyophilized and diluted with Na-phosphate buffer containing 1 mM β-mercaptoethanol (β-ME) to a final concentration of 80 μM. The measurements were conducted in the absence and presence of biotin (~3:1 molar ratio, biotin: bradavidin II subunit). Avidin was used as a control and treated likewise. The thermograms (25–130 °C, 0.92 °C/min) were collected with a Nano II high-sensitivity differential scanning calorimeter (Calorimetry Sciences Corp., Lindon, UT, USA). CpCalc 2.1 (Calorimetry Sciences Corp.) and Microcal Origin 6.0 (MicroCal, Northampton, MA, USA) were used for data analysis.

A thermal stability assay was conducted by subjecting bradavidin II to heat in the absence and presence of biotin at the indicated temperatures for 20 min [17]. The concentration of bradavidin II was 25 μM (1 M PBS, pH 7.4), that of biotin 0.7 mM. An equal volume of SDS-PAGE buffer was added prior to heating. The analyses were made in the presence of biotin at 25, 50, 60, 70, 80 and 90 °C, and in the absence of biotin at 25, 40, 50, 60, 70 and 80 °C. After heat treatment the samples were analyzed in 15% SDS-PAGE gel followed by Coomassie Brilliant Blue staining.

The activity (in the sense of binding biotin) of heat-treated and immobilized protein was studied by ELISA as previously described [18]. Bradavidin II was diluted with a sodium phosphate buffer (50 mM Na-phosphate, 100 mM NaCl, pH 7) to a final concentration of 0.2 μM. The samples were heated at 80, 90 and 100 °C for 0–30 min, followed by incubation on a Nunc MaxiSorp™ microplate at 4 °C overnight. The plate was washed 3 times with 300 μl of PBS-BSA (1% BSA) and blocked with the same buffer for 30 min at 37 °C. Biotinylated alkaline phosphatase (50 μl, 1 mg/ml) was added in each well, incubated for 60 min at 37 °C and washed as described above. *para*-Nitro phenyl phosphatase (pNPP, 1 mg/ml, 1 M diethanolamine, pH 9.8) was added and the activities of the proteins were measured immediately after the addition by an ELISA plate reader at 405 nm for 60 min in 5 minute time intervals [19].

2.8. Ligand-binding analysis

An isothermal titration calorimetry VP-ITC MicroCalorimeter (MicroCal LLC, Northampton, MA, USA) was used to study bradavidin II interactions with D-biotin. The protein sample was dialyzed against sodium phosphate buffer (50 mM Na-phosphate, 100 mM NaCl, pH 7) and diluted to a final concentration of 40 μM. Biotin was diluted with the same buffer to a final concentration of 0.4 mM. MicroCal™ ThermoVac was used to degas and heat the samples to 24 °C for 5 min with stirring. The experiments were conducted at 25 °C, using a 10 μl titration aliquot, a constant stirring speed of 330 rev/min and 200-s spacing. The data were analyzed with Microcal Origin 7.0 (MicroCal LLC, Northampton, MA, USA, 2002) software. ITC was used to determine the heat evolved in the binding process.

The dissociation rate constant (k_{diss}) of bradavidin II was determined by the D-[8,9-^{3}H]-biotin (Amersham Biosciences, Little Chalfont, UK) displacement method at 20, 30 and 40 °C. D-[8,9-^{3}H]-biotin (2 μl) was mixed with buffer (4 ml, 50 mM Na-phosphate, 100 mM NaCl, 0.1% BSA, pH 7). A sample (300 μl) was moved to a filter (Microcon YM-30, Millipore Corporation, Bedford, MA, USA) and centrifuged (2 min, 13,000 *g*). Three samples (30 μl) were collected from flow-through, added in 4 ml of OptiPhase 'HiSafe' 3 (Perkin Elmer Life and Analytical Sciences B. V., Groningen, The Netherlands) and the radioactivity of D-[8,9-^{3}H]-biotin was measured. Bradavidin II (50 nM protein, 50 mM Na-phosphate, 100 mM NaCl, pH 7) was added into the remaining buffer solution (3.7 ml) and incubated for 10 min at indicated temperature. The dissociation reaction then initiated by adding an excess of non-radioactive biotin (15 mM, 11.5 μl) (50 mM Na-phosphate, 100 mM NaCl, 0.1% BSA, pH 7). Samples were collected after 2, 5, 10, 20, 40 and 60 min and 2, 18 and 24 h incubation. Unbound and displaced biotin was separated by filtration as described earlier and the amount of displaced D-[8,9-^{3}H]-biotin being detected by a scintillation counter (Wallac 1410, Gaithersburg, MD, USA). The dissociation rate constant (k_{diss}) was calculated from a fitted slope of a line from a plot of ln (fraction bound) versus time according to Klumb et al. [20].