Detailed characterization of the solution kinetics and thermodynamics of biotin, biocytin and HABA binding to avidin and streptavidin

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Abstract

The high affinity (K_D ~ 10^{-15} M) of biotin for avidin and streptavidin is the essential component in a multitude of bioassays with many experiments using biotin modifications to invoke coupling. Equilibration times suggested for these assays assume that the association rate constant (k_{on}) is approximately diffusion limited (10^{9} M^{-1}s^{-1}) but recent single molecule and surface binding studies indicate that they are slower than expected (10^{5} to 10^{7} M^{-1}s^{-1}). In this study, we asked whether these reactions in solution are diffusion controlled, which reaction model and thermodynamic cycle describes the complex formation, and if there are any functional differences between avidin and streptavidin. We have studied the biotin association by two stopped-flow methodologies using labeled and unlabeled probes: I) fluorescent probes attached to biotin and biocytin; and II) unlabeled biotin and HABA, 2-(4'-hydroxyazo-benzene)-benzoic acid. Both native avidin and streptavidin are homo-tetrameric and the association data show no cooperativity between the binding sites. The k_{on} values of streptavidin are faster than avidin but slower than expected for a diffusion limited reaction in both complexes. Moreover, the Arrhenius plots of the k_{on} values revealed strong temperature dependence with large activation energies (6–15 kcal/mol) that do not correspond to a diffusion limited process (3–4 kcal/mol). Accordingly, we propose a simple reaction model with a single transition state for non-immobilized reactants whose forward thermodynamic parameters complete the thermodynamic cycle, in agreement with previously reported studies. Our new understanding and description of the kinetics, thermodynamics, and spectroscopic parameters for these complexes will help to improve purification efficiencies, molecule detection, and drug screening assays or find new applications.
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Introduction

The extremely high affinity of biotin (B7, vitamin H) for avidin (AV) and streptavidin (SAV) is widely exploited in biotechnology and biochemistry in a vast array of applications [1, 2]. It has been used in molecular biology as markers to identify functional moieties in proteins and receptors [3], and the development of bioprocessing affinity chromatography columns for the recovery of highly valued biomolecules [4]. More recently, advances in the characterization of these complexes have allowed the development of highly specific immunoassays, biosensors, and “omic” tools for disease identification and molecular mechanism elucidation [5–8]. Furthermore, B7 and avidin-like interactions can be exploited for imaging purposes in the development of assays (such as, in-vivo real-time visualization of intracellular or other type of biological processes [9, 10]), and for monitoring the delivery of small molecules, proteins, vaccines, monoclonal antibodies, and nucleic acids in nanoscale drug delivery systems [11]. SAV and B7 are used in Fluorescence Resonance Energy Transfer (FRET) [12] systems for drug High Throughput Screening (HTS) applications, commercially known as Homogeneous Time-Resolved Fluorescence (HTRF) [13–15]. Additionally, it has been suggested that these proteins function in nature as antimicrobial agents by depleting B7 or sequestering bacterial and viral DNA [16, 17]. Questions concerning the biological importance have been appeared, as more avidin-like proteins are discovered in other species; for example, rhizavidin was discovered from proteobacterium Rhizobium etli [18, 19], tamavidin from the basidiomycete fungus Pleurotus cornucopiae [20], xenavidin from the frog Xenopus tropicalis [21], bradavidin from Bradyrhizobium japonicum [22, 23]; genes encoding for avidin related proteins have been found in chicken, Gallus gallus, and studied as recombinant proteins [24–31].

The monomers of AV and SAV are eight stranded anti-parallel beta-barrels with several aromatic residues forming the B7 binding site at one end of the barrel [32]. Two monomers lie parallel to each other forming a dimer with an extensive interface and two dimers associate forming the weaker interface of the homo-tetramer. The unbound tetramer has modest thermal stability and the protein becomes highly thermal stable with ligand bound [33]. Intriguingly, the dimeric interface appears to be necessary for high affinity as two interface mutations show interesting effects on the K_D, a Trp110 to Lys mutation causes dimers of high affinity to form, and an Asn54 to Ala mutation results in only monomers, that remains monomeric with ligand bound, with a significantly reduced affinity (K_D ~ 10^-7 M) [34]. Thus, the use of monomeric avidin in affinity chromatography allows for reversible binding.

As it can be inferred, new applications for AV-B7 related complexes will surely continue to emerge as more derivatives are characterized. However, to obtain reliable and sensitive applications, a better understanding of the thermodynamics, fluorescence behavior of the attached probes, and kinetic reaction mechanisms of B7 and avidin-like systems are surely needed. This information can be used to improve purification efficacies, detection, drug screening assays, and to develop new nanotechnological applications. Therefore, we want to provide a more global description of the AV-B7 and SAV-B7 systems for bio- and nano-technological applications.

The association rate constant (k_on) of B7 binding to AV has been assumed to be near diffusion limited since it was first measured by Green [35] (7.0 × 10^7 M^-1 s^-1, pH 5 and 25˚C) employing a quenching experiment that required the quantification, by chromatographic separation, of un-reacted ^14C-biotin. Since then several widely varying k_on values have been reported for both AV and SAV ranging from 1 × 10^5 M^-1 s^-1 to 2 × 10^8 M^-1 s^-1 [20, 36–39] with error ranges below 10%.

Despite this information, the kinetic and thermodynamic parameters of the B7 association to these AV and SAV proteins have not been studied with systematic detail. Consequently, for
In this study, we asked whether the association rate constants ($k_{\text{on}}$) for B$_7$ binding to AV and SAV are truly diffusion controlled, what the association model and thermodynamic cycle that describe the reaction process are, and if there are any functional differences between AV and SAV. In this sense, we analyzed the $k_{\text{on}}$ for B$_7$ binding to AV and SAV by two stopped-flow (SF) methodologies employing fluorescent dye labeled- and unlabeled-B$_7$ derivatives. In the first case, the association reactions were monitored with two sensing modalities: fluorescence change, F(t), and corrected fluorescence anisotropy, rF(t), under pseudo-first-order conditions as a function of temperature, concentration, and pH with the help of three dye-labeled B$_7$ probes: 1) biotin-4-fluorescein (BFl), 2) Oregon green 488 biocytin (BcO), and 3) biotinylated DNA labeled at the 3' end with fluorescein (B$_7$-DNA$_{ds}$-Fl-3') (Fig 1). The functional cofactor form of B$_7$ is biocytin (Bc) which is formed through an amide linkage between the $\varepsilon$-amine of lysine and carboxyl group of B$_7$. Modified BcO contains a significantly longer linker with respect to BFl which allows analysis of a potential steric effect in the association process, as has been reported elsewhere [40].

We also studied the effect of AV glycosylation by enzymatically removing the carbohydrate motif to compare the respective association rates with those of the untreated AV, SAV and analogous probes in other studies [20, 36–39]. To track bound tetrameric species that appeared after SF mixing at pseudo-first order reaction conditions, we show that the binding polynomial

**Fig 1. Dye-labeled B$_7$ probes.** (A) Biotin-4-fluorescein (BFl) contains a shorter spacer of 10 non-hydrogen atoms between the bicyclic ring and the dye structure. (B) Oregon green 488 Biocytin (BcO) has a spacer of 20 non-hydrogen atoms between the bicyclic ring and the fluorescent dye. Biocytin (Bc) is an amide formed with B$_7$ and L-lysine. (C) biotinylated DNA labeled at the 3' end with fluorescein (B$_7$-DNA$_{ds}$-Fl-3'), where B$_7$ was attached to a 14-mer DNA duplex labeled with fluorescein (Fl) at the 3' end with 16 non-hydrogen atoms between the bicyclic ring and the thymine cyclic base. Unlabeled B$_7$ was used to find the reaction rate of the final binding site in AV and compare it with the reaction rates of the initial binding site to assess possible cooperativity.
distribution (Z) allows us to know the fraction of unbound protein, and protein binds to one, two, three and four B7 molecules. Thus, we make a distinction of the AV and SAV complexes using a simple filling model AB_n where A is either AV or SAV, and “n” is the total available number of binding sites occupied by the dye-labeled B7 probes and not the Hill number associated with cooperative binding.

For the second methodology, using a relaxation kinetics approach, the association reactions of unlabeled B7 were monitored in SF instrumentation by tracking the absorbance changes of an AV-HABA complex as B7 replaces bound HABA [41]. The presence of ligand stabilizes the avidin tetramer. AV-HABA relaxation experiments were used to determine if stabilizing the tetramer affects the association rate constants and cooperativity.

Global fitting of the kinetic traces and reported calorimetry values allowed us to test reaction models and discriminate the most probable reaction mechanism, as carried out in previous studies [42–45]. Consequently, the respective activation energies calculated by Arrhenius plots of association rates allowed the acquisition of the forward thermodynamic parameters toward the transition state: enthalpy (E^*_{forward} or ΔH^*_{forward}), entropy (ΔS^*_{forward}) and Gibbs energy (ΔG^*_{forward}) of AV and SAV activated complexes. The forward thermodynamic data is in excellent agreement with the backwards thermodynamic values calculated with the dissociation rate constants (k_{off}) reported by N. M. Green in his seminal work [35]. Additionally, we explain the nature of the second dissociation phase first observed and correctly neglected by Green as a bimolecular “displacement” rate constant (k_{displacement}_{off}), in addition to the detection of the documented unimolecular “replacement” rate constant (k_{replacement}_{off}) [26, 35] which is used to establish the well-known dissociation constant, K_D, as the most stable complex in nature.

Furthermore, we studied the changes in fluorescence lifetime (τ), quantum yield (QY), dynamic quantum yield (Φ), dye emitting fraction (1-S) and steady state anisotropy (r_{ss}) of the fluorescent probes before and after complex formation. These spectroscopic properties provide indications of the chemical environment surrounding the B7 binding pocket in AV and SAV and have important relevance in fluorescence assay detection limits as the signal to noise ratio can be improved by carefully choosing linker length and fluorescent probe.

**Experimental procedures**

**Materials**

**Probes and solution conditions.** Oregon green 488 Biocytin (lot 40300A, Fig 1) was purchased from Invitrogen (Eugene, OR). Avidin (CAS 1405-69-2, lot 608540) was purchased from Calbiochem (La Jolla, CA). HABA or 2-(4’-hydroxyazobenzene)-benzoic acid (CAS 1634-82-8, lot 52F-0073), streptavidin (CAS 9013-20-1), endoglycosidase H (CAS 37278-88-9) and d-biotin (CAS 58-85-5, lot 13F-3199) were all purchased from Sigma Aldrich (St. Louis, MO). Biotin-4-fluorescein (lot 31005, Fig 1) was purchased from Biotium, Inc. (Hayward, Ca). The 3’ end labeled fluorescein top strand with a modified biotinylated d-thymine at position 6 in the following sequence: 5’-GGGAA(biotin-dT)AACTTGGC-3’ (Fig 1) and the respective complement (5’-GCCAAGTTATTCCC-3’) were made by Tri-Link Biotechnologies, Inc. (San Diego, CA), and were both HPLC and PAGE purified. The sequences retain the G/C (base pairs) ends and fluorescein identical to those characterized extensively in our previous studies [42, 44, 46]. The biotinylated 14mer duplex (B7-DNA_ds^*Fl) was formed with 5-10X excess complement and incubated for at least 20 min.

**Protein and active site concentrations.** The AV and SAV concentrations were determined with the HABA colorimetric assay of Green [40] for which absorbance measurements, with total protein at 280 nm (1.54 = 1 mg/ml) and HABA at 500 nm (35500 M^{-1} cm^{-1} bound,
480 M"^(-1)cm"^(-1)(unbound) were made with a Cary 300 Bio UV-Vis spectrophotometer (Varian Inc., Palo Alto, CA). The occupancy of the dye-labeled probes on the AV and SAV tetramer ("p") was obtained with the expansion version of the normalized partition function, \( Z = (p + q + x)^4 \). In considering the totality of binding sites in the AV and SAV tetramer, let "p" denote the fraction of total sites occupied by B_7 ligands (or HABA), "q" the fraction that are unoccupied and are available for binding, and "x" the fraction that are unavailable. The normalized partition function that describes the mole fractions of the various possible AV and SAV tetrameric species is given by \( Z = (p + q + x)^4 \); where "x", from the HABA assay for AV, was found to be 0.185 (or 18.5%), and \( q = 1 - p - x \). Knowing the total concentration of binding sites from UV protein absorbance and Green's methodology [40], and determining "x", results in the maximum value of "p" that will be reached in reacting tetramers with a B_7 analog. Expansion of \( Z \) provides the mole fractions of the various species in solution, and in decreasing order in terms of probe occupancy, are: \( p^4 + 4p^3q + 6p^2q^2 + 4pq^3 + 4p^2q^2 + 12p^2qx + 4pq^3 + 4px^3 + 12pq^2x + 12pq^2x + q^4 + x^4 + 4q^2x + 4q^2x + 6q^2x^2 \) which totals 1. This development assumes completely random occupancy of probe and inactive sites characterized by "x". The species containing one bound probe have "p" raised to the first power; those with two bound probes have "p" raised to the second power, and so on.

All of the following protein concentrations are presented on a binding site basis, thus in the case of the HABA association reactions for AV were measured at 23.0 ± 0.1˚C with a concentration of 87 μM HABA and 7.7 μM AV. The AV-HABA relaxation reactions were conducted with a preformed AV-HABA complex made up of 200 μM HABA and 10 μM AV, flowed against varying amounts of B_7 from 100 μM up to 4000 μM for a \([\text{HABA}]/[\text{B}_7]\) ratio that ranged from 0.05 to 2.

**Association stopped-flow kinetics.** These reactions were carried out in a buffered solution of 10 mM Tris-HCl, 100 mM KCl, 2.5 mM MgCl_2 and 1 mM CaCl_2 at pH 8 and only AV-BcO reactions included pH 9 and 10. The concentrations, after mixing, were of 20 nM of dye-labeled B_7 probe and 260 nM, 520 nM or 1040 nM of AV; and 200 nM, 300 nM, 400 nM or 800 nM of SAV at temperatures of 10, 15, 20 and 25˚C. The deglycosylation of AV (for comparative association reactions) was carried out using the provided standard protocol with endoglycosidase H [47], both with and without incubation of a denaturant solution (2% SDS and 1M 2-mercaptoethanol).

**Dissociation reactions of dye-labeled biotin complexes.** Biotin dissociation was determined using labelled biotin (BcO and BFl) displaced by unlabeled biotin using minimally occupied and fully occupied binding sites. In the minimally occupied measurements, SAV is prepared with less than one site on average occupied by labelled biotin (AB_1), using 800 nM SAV and 40 nM of BcO or BFl. For saturated SAV-labelled biotin (AB_4) complexes, equimolar binding sites and labelled ligand were prepared, 40 nM SAV and 40 nM of BcO or BFl. The AB_1 complexes were challenged in displacement experiments with several concentrations of unlabeled B_7 (1500 nM, 1750 nM, 2000 nM and 2500 nM) at 20 ± 0.1˚C. In AB_4, SAV had 760 nM in open sites, therefore the total challenging B_7 concentrations were 740 nM, 990 nM, 1240 nM and 1740 nM, respectively. Additional measurements at 27 ± 0.1˚C using 1300 nM, 1500 nM, 1750 nM, 2000 nM and 3000 nM biotin were completed. The 40 nM AB_4 complexes were challenged with unlabeled B_7 concentrations of 400 nM (10X) and 1600 nM (40X) at 20 ± 0.1˚C. The dissociation reactions of AV complexes were carried out with a preformed complex of 20 nM BFl or BcO and 260 nM AV for a filling model of AB_1 and challenged with unlabeled B_7 at 2,000 nM.

**Spectroscopic properties.** The lifetimes (\(\tau\)), steady state anisotropies (\(r_{ss}\)), time-resolved anisotropies (\(r_t\)) and quantum yields (QY) of the complexes (at 20 ± 0.1˚C and pH 8) were collected with a dye-labeled B_7 probe concentration of 20–40 nM and 1040–2080 nM of either
protein (AV or SAV) to ensure that only one binding site in the tetramer was filled with a ligand (AB₁ filling model).

**Methodologies**

The following experiments were carried out by at least six times, unless indicated, and the reported errors correspond to the standard deviation.

**Steady-state anisotropy (r<sub>ss</sub>).** The r<sub>ss</sub> measurements were collected using the Giblin-Parikhurst modification of the Wampler-Desa method as described previously [48]. The fluorescence signal was detected in a model A-1010 Alphascan fluorimeter (Photon Technologies, Inc., Birmingham, NJ) equipped with an R928 PMT (Hamamatsu, Bridgewater, NJ). The excitation was provided by an Ar<sup>+</sup> ion laser (Coherent Innova 70–4 Argon, Santa Clara, CA) at 488 nm and 5–10 mW of power incident on the sample. A photoelastic modulator (PEM-80; HINDS International, Inc., Portland, OR) was placed between the laser source and the sample compartment with a retardation level of 1.22π, and the PEM stress axis orientated 45˚ with respect to the E vector of the laser beam. Two signals were acquired with the PEM alternating between “on” and “off” positions for 10 seconds and the data fitted to a least squared straight line to minimize noise. A minimum of six of these independent measurements were averaged to acquire the r<sub>ss</sub> values. The fluorimeter G factor was determined using a film polarizer and analyzer with an excitation at 488 nm provided by a xenon arc lamp (model A1010, Photon Technologies Inc, Princeton, NJ). The dissociation reactions of dye-labeled B<sub>7</sub> and protein complexes were monitored by fluorescence changes and were also collected in the fluorimeter described above.

**Fluorescence lifetimes (τ) and time-dependent anisotropy decays (r<sub>t</sub>).** The lifetimes were collected in a FluoTime100 fluorescence spectrometer (PicoQuant, GmbH, Berlin, Germany) with the excitation light source provided by a picosecond pulsed diode laser (PicoQuant, GmbH, Berlin, Germany) at 470 nm and 20 MHz. The emission was collected at 520 nm through a non-fluorescing 520 nm interference filter (Oriel Corp., Stratford, CT) followed by a liquid filter of 1 cm path length containing 24 mM acetate buffered dichromate at pH 4, between the sample and detector to eliminate traces of excitation light [42]. The fluorescence decays were fit by a nonlinear least-squares minimization based on the Marquardt algorithm embedded in the Fluofit software (PicoQuant GmbH). Twenty-eight decays were collected per sample, the decays were grouped in four sets, consisting of seven sample decays and one Instrument Response Function, IRF, for deconvolution proposes. The decay sets were globally fitted to mono- or bi-exponential decay models that were discriminated using the statistical parameter χ². The r<sub>t</sub> data were acquired with the fluorimeter described above equipped with a polarizer and an analyzer to acquire the parallel VV(t) and perpendicular VH(t) decays. The PicoQuant G factor was calculated according to: G = \( \frac{\int V(t) dt}{\int H(t) dt} \), where HV(t) and HH(t) were the decays collected with the emission polarizer selecting vertical and horizontal E-vector passing orientations, respectively, and the excitation polarizer set at horizontally position.

**Quantum yields (QY).** The QY values were obtained by using a reference fluorophore of known quantum yield and were calculated according to Parker and Rees [49, 50], where the reference dye was fluorescein in 0.1N sodium hydroxide solution [46]. The emission fluorescence scans were collected from 480 nm to 700 nm with the excitation light set at 460 nm provided by the xenon arc lamp described above. These measurements were made on the AB₁ complexes at high protein concentration.

**Intrinsic lifetime (τ<sup>ᵢ</sup>), dynamic quantum yield (Φ) and fraction of non-statically quenched molecules (1-S).** These calculations have been described elsewhere [46] and were
acquired for the AB₁ complexes. The HABA association reaction for AV was carried out under pseudo-first order conditions on a micro absorbance SF instrument [51] equipped with a xenon arc lamp (described above) and a monochromator (model 82–410, Jarrel-Ash, Waltham, Mass.) set at 500 nm.

**Relaxation kinetics of unlabeled biotin reacting with the AV-HABA complexes.** The relaxation experiments were prepared at concentrations in which HABA occupies all sites (AV-HABA₄). Biotin replaces HABA relative to the \( k_{\text{off}} \) of the dye as shown for the first step (Eq 1) and then repeated for all sites. Having greater affinity, B₇ replaces HABA relative to the experimental conditions, forming a unoccupied site that remains acquired for the AB₁ complexes. The HABA association reaction for AV was carried out under pseudo-first order conditions on a micro absorbance SF instrument [51] equipped with a xenon arc lamp (described above) and a monochromator (model 82–410, Jarrel-Ash, Waltham, Mass.) set at 500 nm.

\[
AV\text{-}HABA₄ + B⁷ \xrightarrow{k_{\text{on}}} AV\text{-}HABA₄ + HABA + B⁷ \xrightarrow{k_{\text{off}}} (B⁷\text{-}AV\text{-}HABA₄) + HABA \quad (1)
\]

The reaction is monitored by the HABA absorbance changes at 500 nm as it is replaced by unlabeled B₇, yielding the relaxation constant of the reaction (Relaxation, Eq 2) which contains information of the B₇ association rate constant of the open binding site, \( k_{\text{on}}^{AV\text{-}HABA₄} \), to form a full saturated complex (AV-HABA₄) and the dissociation rate of that full complex, \( k_{\text{off}}^{AV\text{-}HABA₄} \), to yield a complex with three HABA molecules (AV-HABA₃). In the subsequent steps, B₇ replaces HABA as the ligand but the release of HABA creates an unoccupied site that remains in the same state. In summary, the experiment was designed to acquire the pseudo-first order association rate constant of B₇ binding \( (k_{\text{on}}^{AV\text{-}HABA₄}) \) to the solely free binding site in a complex occupied by three HABA molecules (AV-HABA₃).

\[
\text{Relaxation} = \frac{k_{\text{on}}^{AV\text{-}HABA₄} \cdot k_{\text{off}}^{B₇} \cdot [B₇]}{[HABA] \cdot k_{\text{off}}^{AV\text{-}HABA₄} + k_{\text{off}}^{B₇} \cdot [B₇]} \quad (2)
\]

The reciprocal of the relaxation constant (1/Relaxation) is plotted vs. the [HABA]/[B] concentration ratio (Eq 3) allowing to calculate: \( k_{\text{on}}^{AV\text{-}HABA₄} \) and \( k_{\text{off}}^{AV\text{-}HABA₄} \) by solving for the intercept \((1/k_{\text{off}}^{AV\text{-}HABA₄}) \) and the respective slope: \( m = k_{\text{on}}^{AV\text{-}HABA₄} / (k_{\text{off}}^{AV\text{-}HABA₄} \cdot k_{\text{off}}^{B₇}) \). The exponential decays were analyzed by the method of Foss [52]. There was no departure from simple first order decay in the relaxation, justifying the use of the following model and equations.

\[
\frac{1}{\text{Relaxation}} = \frac{k_{\text{on}}^{AV\text{-}HABA₄} \cdot [HABA] + 1}{k_{\text{off}}^{AV\text{-}HABA₄} \cdot [B₇]} + \frac{1}{k_{\text{off}}^{AV\text{-}HABA₄}} = m \cdot \frac{[HABA]}{[B₇]} + \frac{1}{k_{\text{off}}^{AV\text{-}HABA₄}} \quad (3)
\]

**Association reactions of dye-labeled biotin and AV (or SAV).** The reactions were collected with the SF instrument, described previously [53, 54]. The fluorescence signal was collected through a 520 nm interference filter (Oriel Corp., Stratford, CT) with a detector time constant and SF dead time of 1 µs and 1 ms, respectively. The excitation light was provided by the Coherent Ar⁺ ion laser (described above) at 488 nm with 15–10 mW of incident power on the reaction cuvette. The laser source was followed by the photo-elastic modulator described above with the axis oriented 45° with respect to the electric vector of the incident light and with the half-wave modulation (50 kHz) set for 488 nm excitation. The demodulation circuitry following the photomultiplier provided a DC(t) and a rectified AC(t) which were converted to digital data by a high-speed digitizer (PCI-5122) from National Instruments (Austin, TX) with 14-bit resolution and 100 MHz bandwidth, through channels 0 and 1. The data acquisition was controlled by LabVIEW (Vr 8) software at a collection rate of 6120 data points/second and stored in spreadsheets. The AC(t) and DC(t) data were baseline corrected before obtaining the...
signal ratio (Eq 4) as a function of time \( \rho(t) \).

\[
\rho(t) = \frac{AC(t)}{DC(t)} = \frac{1.5 \cdot r(t) \cdot A_{\text{gain}}}{1 - 0.47818 \cdot r(t) \cdot (1 + 2.3806 \cdot H)}
\] (4)

The constant \( A_{\text{gain}} \) is the instrumental amplitude gain and was evaluated by solving \( \rho(t) \) using the known steady state anisotropy \( r_{ss} \) of the complexes which is equivalent to the \( r(t) \) at \( t = \infty \); and \( H \), obtained from the equivalent grating factor \( G \) for the filters and photo multiplier tubes in the SF. For the probes used in here \( G = 0.82 \) and \( H = (1-G)/(1+G) = 0.099 \).

Knowing \( A_{\text{gain}} \) and \( H \), the AC(t) and DC(t) signals can be solved for \( F(t) \) and \( F(0) \) (Eqs 4 and 5) and the normalized fluorescence, \( \bar{F}(t) \), and corrected fluorescence anisotropy, \( \bar{r}F(t) \) \([55]\), were obtained when \( \bar{F}(0) \) and \( \bar{r}F(0) \) were scaled to 1 at \( t = 0 \).

\[
F(t) = \frac{DC(t)}{1 - 0.47818 \cdot r(t) \cdot (1 + 2.3806 \cdot H)}
\] (5)

The \( \bar{F}(t) \) (Eq 6) is equivalent to \( (I_d^0 + 2 \cdot I_u^0) \) and proportional to quantum yield \( (QY_i) \), molar absorptivity \( (\varepsilon_i) \) and to the formation or disappearance of the emitting species \( X_i(t) \); and \( \bar{r}F(t) \) including the steady state anisotropies \( r_{ss} \) of each fluorescent species (Eq 7) \([55]\).

\[
\bar{F}(t) = \sum \varepsilon_i \cdot QY_i \cdot X_i(t) / \sum \varepsilon_i \cdot QY_i \cdot X_i(0) = F(t)/F(0)
\] (6)

\[
\bar{r}F(t) = \sum \varepsilon_i \cdot r_{ss} \cdot QY_i \cdot X_i(t) = rF(t)/rF(0)
\] (7)

**Biotin association reaction model for AV and SAV.** The possible reaction models were discriminated by the squared residuals of the observed and calculated association traces of both fluorescence and anisotropy fluorescence signals, \( F(t) \) and \( \bar{r}F(t) \), respectively. For the BFl and BcO probes, the association reactions were very well described by the simplest possible model (Eq 8) with single association rate constants \( (k_{on}) \).

\[
AV(\text{or SAV}) + \text{Dye–labeled B}_{7}^{\text{ds}} \xrightarrow{k_{on}} \text{Complex}
\] (8)

In the case of the \( B_7^{\text{ds}} \)-DNA\( ds \)–Fl, the association reaction model was complemented by a second \( k_{on} \) which resulted in a system of two parallel reactions (Eq 9). In both cases, the backward reaction is not significant during the 5–8 sec required for the \( B_7 \) association binding.

\[
\begin{align*}
AV(\text{or SAV}) + (B_7^{\text{ds}} \cdot \text{Fl})_1 \xrightarrow{k_{\text{off}}} & \text{Complex} \\
AV(\text{or SAV}) + (B_7^{\text{ds}} \cdot \text{Fl})_2 \xrightarrow{k_{\text{off}}} & \text{Complex}
\end{align*}
\] (9)

**Dissociation reactions of the complexes.** The dissociation reactions were followed by fluorescence changes, \( F(t) \), in the fluorimeter and laser setup described above and tuned to 488 nm under discontinuous excitation to prevent photobleaching distortion. The signal was best fitted to the following dissociation model (Eq 10), in which the dye labeled complex dissociates into the labeled \( B_7 \) probe (BFl or BcO) and the respective protein (AV or SAV).

\[
\begin{align*}
\text{Complex (dye – labeled B}_7\text{)} + B_7 & \xrightarrow{k_{\text{off}}^{\text{displacement}}} AV - B_7 (\text{or SAV - B}_7) + \text{Dye–labeled B} \\
\text{Complex (dye – labeled B}_7\text{)} + B_7 & \xrightarrow{k_{\text{off}}^{\text{replacement}}} \text{AV} - B_7 (\text{or SAV - B}_7) + \text{Dye–labeled B}
\end{align*}
\] (10)

**Time-resolved anisotropy \( (r_t) \).** The \( r_t \) values were calculated according to Eq 11 where the pre-exponential “\( f \)” corresponds to the slow phase that derives from the lifetime of the
global motion \((\tau_G)\) [56] which was fitted within a range of expected correlation time for the complex size [57]; consequently, facilitating resolution of the fast correlation lifetime \((\tau_p)\) and the corresponding pre-exponential \((1-f)\).

\[
\tau(t) = (1 - f) \cdot \exp\left(\frac{-t}{\tau_p}\right) + f \cdot \exp\left(\frac{-t}{\tau_G}\right)
\]  

(11)

The \(f\) parameter was constrained to the observed \(r_{ss}\) (Eq 12) where \(\hat{F}(t)\) (Eq 13) is normalized \((\alpha_1 + \alpha_2 = 1)\) and derived from the observed fluorescence decays of the complex [58].

\[
r_{ss} = 0.4 \int \tau(t) \cdot \hat{F}(t) dt
\]  

(12)

Where

\[
\hat{F}(t) = \frac{\alpha_1 \exp\left(\frac{-t}{\tau_1}\right) + \alpha_2 \exp\left(\frac{-t}{\tau_2}\right)}{\alpha_1 \tau_1 + \alpha_2 \tau_2}
\]  

(13)

In a simple model, the transition moment is assumed to wobble within a cone of semi-apical angle \(\Omega\) [59], where the cone axis is normal to the surface of a sphere that corresponds to the macromolecule. The angle \(\Omega\) is calculated from Eq 14.

\[
f = \left[\frac{1}{2} \cos \Omega (1 + \cos \Omega)\right]^2
\]  

(14)

Results and discussion

Active avidin binding sites

The avidin and streptavidin proteins are tetramers in solution. If the binding of the ligand is positively cooperative, differences in \(k_{on}\) for initial and final binding steps could be significant; therefore, the comparison of initial binding by nonliganded AV and final binding by liganded AV is necessary. Measurement of the initial binding rate requires ligand free AV, but endogenous ligand could potentially interfere. In fact, AV preparations often present about 20% of the inactive sites for the binding of any B\(_7\) analogs, either because they contain endogenous B\(_7\) [40], or perhaps the existence of damaged binding sites in some of them, e.g., tryptophan oxidation [60]. To acquire accurate \(k_{on}\) values, the actual available binding site concentration for each sample was measured by HABA colorimetric assays in relation with absorbance at 280 nm. Accordingly, the percentage of available active sites of AV and SAV were 81.5 ± 1.0% and 94.0 ± 1.0% with respect to total protein, respectively, which were in excellent agreement with the 82% and 95% reported by the commercial source (Sigma Aldrich and CalBiochem). The SF apparatus provided rapid thorough mixing of the probes with AV and SAV allowing measurement of the full reaction. The issue of rapid mixing vs. more conventional titrations was treated previously [51]. In the SF association measurements, the dye-labeled B\(_7\) probes were sub-stoichiometric to determine the initial binding rates (e.g. 20 nM of BFI, BcO and B\(_7\)-DNA\(_{ds}\) vs. 260 nM, 520 nM and 1040 nM in binding sites basis). Limiting the ligand also reduced several potential measurement artifacts including FRET self-transfer, and contact interference including probe fluorescence quenching by contact interactions [61] in the AB\(_2\), AB\(_3\) or AB\(_4\) complexes; especially for the BcO which has a longer linker [62]. Using the binding polynomial for the 20 nM probe after mixing, and 638 nM in total sites for the intermediate AV concentration which corresponds to 520 nM in available sites, the mole fraction of species with a single bound probe is 0.114, that with two bound probes is 0.0055, and with three bound probes is 0.0001, so at most, only 0.55% of the molecules with bound AV contain two
probes; for 1040 nM available sites, the value drops to 0.15% (S1 File). With limited occupancy, the association reactions acquired the dye-labeled Bγ probes reflect the binding to the first binding site in the tetramer for the SF experiments. The unlabeled Bγ relaxation kinetic experiment was designed to observe the binding at the final site, as discussed below.

**Association rate constants (k_{on}) of biotin binding to avidin**

**Dye-labeled biotin association rate constants by stopped-flow methodology.** The fluorescence \( F(t) \) and corrected anisotropy association binding traces, \( \overline{rF}(t) \), properly monitored the association reactions, as they yielded equivalent \( k_{on} \) values (Table 1) and presented the best optimal fit residuals (Fig 2). In contrast, the anisotropy signal, \( r(t) \), lagged behind \( F(t) \) and \( \overline{rF}(t) \) since changes in the quantum yield (QY) of the involved fluorescence species distort the kinetic traces [55]. These three types of association binding traces were acquired with discontinuous excitation that circumvented photobleaching (Fig 3) allowing the detection of all non-photobleaching rate constants. Consequently, the \( k_{on} \) values of AV showed linear concentration dependence (Fig 4) and strong temperature dependence when using the BcO (Fig 5) and BFl (Table 2) probes. Notably, a reduction in the \( k_{on} \) of ~10% was observed with each pH unit increment (from 8 to 10) which may derive from titration of the hydrogen bonding of asparagine and tyrosine in the binding pocket [32].

**Unlabeled biotin association rate constants by relaxation kinetics methodology.** The experiment consisted in challenging a pre-saturated AV-HABA complex with Bγ (Fig 6) to measure the association rate of the final “relaxed” binding sites which yielded a \( k_{on} \) of 5.3 ± 0.9 × 10^6 M⁻¹s⁻¹ (at pH 8 and 23°C) which is slightly slower than the 7.8 ± 0.4 × 10^6 M⁻¹s⁻¹ acquired with BcO (Arrhenius plot, 23°C and pH 8) indicating non-cooperativity (or slightly negative) for binding site association rates. The HABA dissociation rate constant of the AV-HABA₄ complex was not rate limiting (\( k_{AV-HABA}^{-1} = 6.23 ± 0.11 \text{ s}^{-1} \)) and the HABA association rate for the final site was \( k_{AV-HABA}^{1} = 5.1 ± 0.1 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) which results in an AV-HABA equilibrium constant of \( K_{D}^{AV-HABA} = 12.2 ± 0.3 \times 10^{-6} \text{ M} \) similar to that reported by Green [60] at pH 8 which supports the quality of our relaxation kinetic experiment.

**Non-cooperative biotin binding to avidin sites.** The association reactions that used the fluorescent probes BFl and BcO monitored the 1st available binding site, as they were carried out at pseudo-first order, at very high protein concentration with low occupancy for the AB₃ filling model, as discussed above. In contrast, the relaxation kinetic methodology scrutinized the unlabeled Bγ binding to the unoccupied site while the 3 remaining sites were filled with HABA, this process can be thought as the binding of Bγ to the 4th binding site. Therefore, the data obtained with dye-labeled Bγ probes and unlabeled Bγ should report the binding rates to

| Temp. | 260 nM | 520 nM | 1040 nM | 260 nM | 520 nM | 1040 nM |
|-------|--------|--------|---------|--------|--------|---------|
| 25°C  | 9.5 ± 0.1 | 9.5 ± 0.3 | 9.7 ± 0.3 | 9.5 ± 0.1 | 9.5 ± 0.1 | 9.4 ± 0.2 |
| 20°C  | 5.7 ± 0.1 | 5.9 ± 0.3 | 6.0 ± 0.1 | 5.9 ± 0.1 | 5.8 ± 0.1 | 6.1 ± 0.1 |
| 15°C  | 4.1 ± 0.1 | 4.0 ± 0.1 | 3.9 ± 0.2 | 4.0 ± 0.1 | 3.8 ± 0.1 | 4.0 ± 0.2 |
| 10°C  | 2.4 ± 0.1 | 2.6 ± 0.2 | 2.5 ± 0.2 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.1 |

The association reactions were acquired with BcO (20 nM) binding to AV at several temperatures, protein concentrations and pH 8. The \( F(t) \) and \( \overline{rF}(t) \) signals were equivalent as they tracked in the errors the association process of dye-labeled Bγ binding to the proteins under pseudo-first order conditions.

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Detailed characterization of avidin, strepavidin, and ligands

Comparisons with other AV-B kinetic studies. Comparisons with other AV-B2 kinetic studies were carried out at the closest possible condition; thus, at 25°C and pH 8, the BFI and BcO association rate constants, $k_{\text{on}}$, were 3.8X and 7.4X slower than the 7X reported by N. M. Green [35] (at 25°C and pH 5), respectively. However, a larger uncertainty is expected for the latter experiment because it was not carried out using rapid mixing techniques forcing the usage of very low (14 carbon) B2 concentrations (picomolar range) to timely stop the reaction and quantify the un-reacted probe. Consequently, Green’s experiment was an extremely tedious task that was carried out, only once and at one temperature. On the other hand, a more recent association rate constant of 2.0 ± 0.3 × 10^6 M^{-1}s^{-1} was obtained in a

**Fig 2. Comparison of the association kinetic traces.** (A) Fluorescence, $F(t)$, anisotropy, $\tau_F(t)$ and corrected fluorescence anisotropy, $\tau_F(t)$ reaction traces of BcO (20 nM) binding to SAV (200 nM) at 10°C. The monoeXponential fits (black) resulted in $k_{\text{on}}$ values of 1.73 × 10^7 M^{-1}s^{-1}, 1.72 × 10^7 M^{-1}s^{-1} and 1.04 × 10^7 M^{-1}s^{-1} with halftimes of 200.6 ms, 201.4 ms and 332.7 ms, respectively. The respective residual of the reaction traces versus fit curves show that (B) $\tau_F(t)$ and (C) $\tau_F(t)$ were optimal, and (D) $\tau_F(t)$ signal is ill-fitted. The $k_{\text{on}}$ of $\tau_F(t)$ was 40% slower than the other two and showed the worst residuals due to changes in QY [55]. The corresponding normalization signals were:

$\tau_F(t) = [F(t) - rF(\infty)]/[rF(0) - rF(\infty)]$ and $\tau_F(t) = [r(0) - r(t)]/[r(0) - r(\infty)]$.

\[ \tau_F(t) = [F(t) - rF(\infty)]/[rF(0) - rF(\infty)] \]

\[ \tau_F(t) = [r(0) - r(t)]/[r(0) - r(\infty)] \]

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the 1st and 4th sites. Since these two values only diverge by 32% we believe that there is not significant cooperativity nor an intrinsic difference in any of the AV sites. If a protein has two forms, denoted as relaxed (R) and tense (T), the HABA bound ligand can hold the AV protein in the R-state [63]. In the relaxation experiments, all the bound HABA gets replaced by dye-labeled B2 (BFI or BcO), but all the sites rest in the R-state; therefore, there is not switching from T to R. This is the same as hemoglobin bound to (HbO2) flowed against CO, where O2 gets replaced by CO but is not biphasic because no T-state is present [63, 64]. As B2 binding to AV and SAV is non-cooperative, the HABA replacement is a pseudo first order measure of the B2 association rate and should be the same or close to the association rate of the dye-labeled B2 flowed against empty AV or SAV. Our values differed only by 32% for these two approaches.

**Fig 3. Photobleaching of BcO binding to AV at 15°C.** The photobleaching rate constant was elucidated with the (A) $\tau_F(t)$ and (B) $\tau_F(t)$ signals by collecting the reaction with continuous (black) and discontinuous (dashed color) laser illumination in which the beam was blocked during the times denoted by dashes and the sample was illuminated only during time intervals of 10 s. The slow photobleaching rate constant varied from 6 × 10^{-3} to 1 × 10^{-2} s^{-1}, and was laser power dependent. The (A) $\tau_F(t)$ and (B) $\tau_F(t)$ normalization functions were: $\tau_F(t) = [F(t) - rF(\infty)]/[rF(0) - rF(\infty)]$ and $\tau_F(t) = [r(0) - r(t)]/[r(0) - r(\infty)]$, respectively.

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Surface Plasmon Resonance (SPR) study [20] at 20˚C and pH 7.4 in HEPES buffer. This independent $k_{on}$ value was ~9X and ~5X slower than the ones acquired by us for BFl and BcO, respectively. Nevertheless, it has been previously acknowledged that the SPR results are, controversially, too low to be accurate [20, 39], due to fixation of one of the reactants to the chip, generally AV or SAV.

Effect of AV glycosylation on the biotin binding kinetics. The AV protein has a glycan attached to asparagine 17 at each tetrameric subunit which is composed of four or five mannoses and three N-acetylgalactosamines [65]. These sugar modifications are typically removed to improve crystallization but the glycan effect on the association binding rate of $B_7$ was previously unknown. Interestingly, after enzymatic removal of the carbohydrates, the $k_{on}$ values of the de-glycosylated AV matched those of natural glycosylated AV for the dye-labeled $B_7$ probes: e.g., $3.7 \pm 0.3 \times 10^{-6}$ M$^{-1}$s$^{-1}$ vs. $3.9 \pm 0.3 \times 10^{-6}$ M$^{-1}$s$^{-1}$ of BcO binding to de-glycosylated AV and untreated AV at 15˚C, respectively. A previous study already suggested that the sugar chain is not required for $B_7$ binding [65] and now we confirm that AV glycosylation has no influence on the association rate constants.

Association reaction of unlabeled and dye-labeled biotin binding to streptavidin

Dye-labeled biotin association reactions to SAV. The SAV-$B_7$ association reactions presented temperature (Fig 4C and 4D) and linear concentration dependence (Fig 5C and 5D) and were faster than those of acquired with AV for both dye-labeled probes. For instance, BFl and BcO at 25˚C, presented $k_{on}$ values when binding to SAV that were 4X and 3.2X faster than those observed when binding to AV, respectively. However, the temperature dependence was weaker than that observed for AV which indicated a profound difference in the binding site properties of these two proteins, as reveled by an Arrhenius plot (see 3.9 Thermodynamic Parameters). Thus, SAV should be a more robust system for purification applications as variations on the temperature incubation protocols have less negative significant effects in the yield.

Comparisons with other SAV-$B_7$ association kinetic studies. An independent SF study tracked the binding of unlabeled $B_7$ by fluorescence quenching of the tryptophan (Trp) of SAV, yielding a $k_{on}$ of $7.5 \pm 0.6 \times 10^7$ M$^{-1}$s$^{-1}$ (at 25˚C and pH 7) [39] which was in excellent agreement with $7.5 \pm 0.2 \times 10^7$ M$^{-1}$s$^{-1}$ for the BFl probe (at 25˚C and pH 8). This finding strongly indicates that the attached dyes are innocuous and dependably monitor the $B_7$ binding to SAV and presumably to AV. In addition, the absence of any detectable intermediate in the association reaction in both cases is remarkable, since we monitored the initial binding of $B_7$ and SAV using the fluorescence change and fluorescence anisotropy signals, and the independent tryptophan–quenching experiments observed the final docking of $B_7$ near the Trp [39]. Conversely, there is another independent Surface Plasma Resonance (SPR) study of immobilized $B_7$ binding to SAV that yielded a slower $k_{on}$ of $5.1 \times 10^6$ M$^{-1}$s$^{-1}$ at 4˚C [66], which was ~5X slower than our $2.6 \times 10^7$ M$^{-1}$s$^{-1}$ at 4˚C, calculated by an Arrhenius plot (ln $k_{on}$ vs 1/T) of the BFl data. Similarly to AV, we believe that the SPR methodology for the $B_7$ and AV-like protein kinetics [20, 39] was modified by the immobilization of one reactant, either $B_7$ or protein, to the chip.

Biotinylated and dye-labeled DNA duplex association reaction to AV and SAV

Association rate constants of $B_7$ attached to biotin-DNA$_{flu}$-Fl. The biotinylated 14-mer duplex association kinetics showed a biphasic behavior with two temperature and concentration dependent rate constants (Table 2, Fig 7) when reacting with both AV and SAV. The
biphasic association rate constants, $k_{\text{on}1}$ and $k_{\text{on}2}$, summed to approximately 70% of the total reaction amplitude. The remaining ~30% was assigned to a third-rate constant (0.02 ± 0.01 s$^{-1}$) that presented neither temperature nor concentration dependence; therefore, it has been assigned to the readjustments of the Fl dye after being displaced by both proteins. The $k_{\text{on}1}$ and $k_{\text{on}2}$ association rate constants of SAV were 3.4X and 1.8X faster than the corresponding rate constants of AV (Fig 8) as observed with the BFl and BcO probes, confirming the differences in the AV and SAV binding pockets.

Comparisons with other biotinylated DNA kinetic studies. An independent FRET study monitored the reaction of B$_7$ attached to the 5’ end of a 46 nucleotide duplex DNA binding to SAV [38]. The reaction also showed two rate constants at pH 8, but at unspecified temperature, pre-exponentials and errors. To make a comparison, we have chosen SAV data at 20˚C whose association rate constant, $k_{\text{on}1}$, of 4.6 ± 0.8 × 10$^7$ M$^{-1}$s$^{-1}$ was in excellent agreement with the 4.5 × 10$^7$ M$^{-1}$s$^{-1}$ reported by the mentioned study. In the case of our $k_{\text{on}2}$ of
2.3 ± 0.1 × 10^6 M^-1 s^-1, it was in good agreement with the second rate of 3.0 × 10^6 M^-1 s^-1 of that independent study. The agreement in the data validates our findings which imply that B7 attached internally to DNA (or at the 5’ end) will have two rate constants, one enhanced and other diminished probably due to unfavorable orientation according to the reaction models discussed below.

**Significance of the association rate constants**

The B7 binding to AV and SAV (at 25°C) were, respectively, between 54-714X and 13-400X slower than 10^9 M^-1 s^-1 as expected for a diffusion limited process [67]. On the other hand, the k_{on} values of SAV were 3-4X faster than AV’s despite the similarity of the AV and SAV binding sites in the crystal structures (Fig 8). Our deglycosylation experiments indicate that the
Table 2. Temperature dependent association rate constants ($k_{on}$) and thermodynamic values of the dye-labeled B$_7$ binding to AV and SAV.

| Complex* | 25$^\circ$C $(\times 10^{5}$M$^{-1}$s$^{-1})$ | 20$^\circ$C $(\times 10^{5}$M$^{-1}$s$^{-1})$ | 15$^\circ$C $(\times 10^{5}$M$^{-1}$s$^{-1})$ | 10$^\circ$C $(\times 10^{5}$M$^{-1}$s$^{-1})$ | $E_a$ Forward (kcal/mol) | $\Delta S^\circ$ (cal/°K mol$^{-1}$) | $\Delta G^\circ$ (kcal/mol) |
|----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|----------------------------|---------------------------------|----------------------------|
| AV-BFl  | 18.5 ± 2.0                      | 10.7 ± 1.6                      | 7.6 ± 2.0                       | 4.86 ± 0.6                      | 14.6 ± 0.6                  | 34.9 ± 2.0                      | 4.2 ± 0.5                      |
| AV-BcO (pH 8)$^b$ | 9.5 ± 0.4                                      | 5.9 ± 0.4                      | 3.9 ± 0.3                       | 2.6 ± 0.3                       | 14.4 ± 0.6                  | 33.0 ± 2.5                      | 4.6 ± 0.3                      |
| AV-BcO (pH 9) | 7.9 ± 0.3 $^c$                                      | 5.4 ± 0.2                      | 3.4 ± 0.1                       | 2.3 ± 0.1                       | 13.9 ± 0.7                  | 30.9 ± 2.5                      | 4.7 ± 0.1                      |
| AV-BcO (pH 10) | 7.2 ± 0.3 $^c$                                      | 4.8 ± 0.2                      | 3.0 ± 0.1                       | 1.9 ± 0.1                       | 14.9 ± 0.7                  | 34.1 ± 1.3                      | 4.7 ± 0.3                      |
| 1) AV-B$_7$-DNAads‘Fl  | 15.7 ± 1.0 (30.5%)$^d$                             | 11.2 ± 0.8 (68.9%)              | 7.4 ± 0.7 (87.5%)               | 4.2 ± 0.5 (95.0%)               | 14.6 ± 0.8                  | 34.8 ± 3.0                      | 4.2 ± 0.2                      |
| 2) AV- B$_7$-DNAads‘Fl | 1.4 ± 0.1 (69.5%)                                      | 0.98 ± 0.05 (31.1%)            | 0.70 ± 0.03 (12.5%)             | 0.52 ± 0.01 (0.05%)             | 14.2 ± 0.5                  | 29.0 ± 0.5                      | 5.6 ± 0.6                      |
| Average | NA                              | NA                              | NA                              | NA                              | 14.4 ± 0.2                  | 32.8 ± 1.2                      | 4.7 ± 0.3                      |
| SAV-BFl  | 74.7 ± 2.0                      | 58.6 ± 1.6                      | 53.1 ± 2.0                      | 45.4 ± 1.0                      | 5.3 ± 0.3                   | 6.6 ± 0.5                       | 3.3 ± 0.6                      |
| SAV-BcO  | 30.3 ± 2.0                      | 24.0 ± 1.6                      | 20.0 ± 1.2                      | 17.3 ± 0.6                      | 6.2 ± 0.4                   | 7.8 ± 0.4                       | 3.9 ± 0.4                      |
| 1) SAV- B$_7$-DNAads‘Fl  | 53.0 ± 1.0 (44.4%)                             | 45.9 ± 0.8 (50%)               | 36.4 ± 0.7 (51%)               | 31.0 ± 0.5 (49%)               | 6.2 ± 0.4                  | 8.8 ± 0.6                       | 3.5 ± 0.5                      |
| 2) SAV- B$_7$-DNAads‘Fl | 2.5 ± 0.1 (55.6%)                                      | 2.3 ± 0.1 (50%)               | 1.7 ± 0.1 (49%)               | 1.50 ± 0.05 (51%)               | 6.2 ± 0.5                  | 3.1 ± 0.3                       | 5.3 ± 0.6                      |
| Average | NA                              | NA                              | NA                              | NA                              | 6.0 ± 0.2                  | 6.6 ± 1.6                       | 4.0 ± 0.6                      |

The forward thermodynamic values ($E_a$ Forward, $\Delta S^\circ$ Forward and $\Delta G^\circ$ Forward) were acquired from global fitting of the rate constants [42, 45] for the most probable model which resulted in a simple reaction with a transition state without intermediates. In the case of the B$_7$-DNA duplex, the reaction model was a two-serial reaction model also with one transition state without intermediates. The nature of the serial reaction is probably caused by two B$_7$ populations with different spatial orientations.

*The probes were BFl, BcO, and B$_7$ attached to a nucleotide in a 14-mer DNA duplex and the respective complex with AV and SAV.

The $k_{on}$ values were averaged from data in Table 1

$^c$The pre-exponentials (in parenthesis) of $k_{on}$ and $k_{off}$ were renormalized after removing a third process associated with remaining photobleaching.

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Disparity in the $k_{on}$ values between both SAV and AV proteins cannot be explained by the presence or absence of the carbohydrate motif on the AV but can be explained by the intermolecular interactions of the aminoacids in the binding pocket and the B$_7$ ring.

Association reactions of biotin vs. biocytin to SAV and AV

In our study, the association rates were acquired with B$_7$ and Bc probes, BFl and BcO; respectively, in which Biocytin presents a longer carbon linker. Interestingly, these $k_{on}$ values only differed by 2-fold (Table 1), from 10$^\circ$C to 25$^\circ$C, when reacting with AV. It is important to clarify that the association rates were not enhanced by the electrostatic attraction of the negative charged probes (BFl and BcO) and the positive AV [32]; since, the association rates of those two probes binding to neutral SAV differed also by ~2 fold as observed for AV. The dissociation constants, $K_{off}$ of AV-B$_7$ and AV-Bc were reported to be 10$^{-13}$ and 10$^{-15}$ M, respectively, differing by 100-fold [40]. Consequently, this 100-fold difference, if accurate, must be caused by a difference of 50-fold in the $k_{off}$, dissociation rate constants which is discussed below.

Dissociation kinetics

The dissociation reactions of the AV-B$_7$ and SAV-B$_7$ complexes have been described as passive unimolecular “replacements” ($k_{off}^{replacement}$) with units of reciprocal seconds (s$^{-1}$) and values of 9 × 10$^{-8}$ s$^{-1}$ [35] and 2.4 × 10$^{-8}$ s$^{-1}$ [68], respectively. However, we have also observed bimolecular “displacement” off-rate constants ($k_{off}^{displacement}$) with M$^{-1}$s$^{-1}$ units for the SAV-BcO

$^d$Calculated from an Arrhenius plot.

$^e$The nature of the serial reaction is probably caused by two B$_7$ populations with different spatial orientations.
complexes (AB$^1$ and AB$^4$) that were strongly dependent on B$^7$ concentration (Fig 9A) and temperature (Fig 9B). These reactions had ~79% of the total release amplitude, in contrast to the 5% when BFl was used (Fig 9C); therefore, the longer "tail" of the BcO facilitated the displacement for SAV-BcO; and in the case of the SAV-BFl, the electrostatic interactions between negative charged Fl and positive charge SAV prevented the displacement, as observed elsewhere [69]. Thus, longer linkers and neutral dye molecules and proteins are features that can be exploited to increase purification yields. This new information can find important applications in affinity chromatography purification based on SAV and longer "tail" or tethers that will help to increase the release of the product and enhance efficiency. On the contrary, we could not detect neither displacement nor replacement in AV-BFl and AV-BcO complexes since the reaction is very slow (S3 Fig). Thus, in 1966, Green N. determined heroically the $k_{\text{replacement}}$ for AV-B7 in $9 \times 10^{-8}$ s$^{-1}$ for a half-life of 90 days [35] which could not be detected by us since our fluorescence anisotropy methodology is not suitable.

**Biotin reaction models of AV and SAV**

**Reaction model of BFl and BcO binding to AV and SAV.** The SF traces of B$^7$ binding to AV and SAV were best fitted by a simple association model, A + B $\rightleftharpoons$ C. A single rate constant, $k_{\text{on}}$ (Eq 8), could be fit with no intermediates or evidence of cooperativity considering that the dissociation reaction was not significant for the first 5–8 sec after mixing. More elaborate mechanisms have been reported [70, 71]. For example, A + B $\rightleftharpoons$ C $\rightleftharpoons$ D has been proposed for polystyrene SAV coated particles (6.5 nM) reacting with a fluorescein labeled B$^7$ probe (1.8 nM and 17.5 nM), whose linker resembles our BcO probe. This model required fitting of two dissociation and two association rate constants with the extra equilibrium attributed to two
Fig 7. Association traces of biotin-DNA\textsubscript{ds}·Fl binding to SAV and AV. The $\overline{r F}(t)$ and $\overline{F}(t)$ signals of the association reactions of B\textsubscript{7}-DNA\textsubscript{ds}·Fl (20nM) to (A) AV (520 nM) and (B) SAV (200 nM), at 15˚C. (C) Concentration dependence of B\textsubscript{7}-DNA\textsubscript{ds}·Fl (20 nM) binding to AV at 15˚C. All curves (black line) were strongly biphasic. Notice the inversion of SF signals. However, the $\overline{F}(t)$ traces were in prefect agreement with QY experiments (Table 4).

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reasons: 1) The interference of the dye structures into the neighboring site due to multiple occupancies on the tetramer [61] and 2) to possible inhibitory steric interactions caused by high density of SAV sites on the surface of the polystyrene particles. Interestingly, a similar model was used to analyze a pull-off study carried out by Scanning Force Microscopy for Fig 8. Arrhenius plot of the association rate constants. Temperature dependence of the B7 association reaction at pH 8 (unless otherwise specified) for: 1 SAV-BFI (purple triangles); 2 SAV-B7-DNA_Fl (green triangles): 2.1 (k_{on1}) and 2.2 (k_{on2}); 3 SAV-BcO (red triangles); 4 AV-BFI (purple circles); 5 AV-B7-DNA_Fl (green circles): 5.1 (k_{on1}) and 5.2 (k_{on2}); 6 AV-BcO (red circles): 6.1 at pH 8, 6.2 at pH 9 (orange circles), 6.3 at pH 10 (yellow circles). The data points were plotted in semi-logarithm (ln k_{on} vs 1/T) for clarity.
AV-B complex with immobilized AV in which two events of 20–40 pico-newtons and 40–80 pico-newtons were assigned to the presence of an intermediate [72]. Categorically, we have avoided these experimental complications by following the reaction at pseudo first order to ensure that our probes occupied only one binding site of AV and SAV in solution (non-immobilized), as discussed above. However, when considering a particular AV or SAV bioassay, one must consider that the surface matrix complexity, the multiple orientations of B-avidin (denoted AV) or SAV (denoted SAV) binding to AV and SAV are only 20–40% slower than rate constants acquired with BFl, which was 740 nM (blue), 1240 nM (pink) and 1740 nM (red) after the remaining free binding sites were filled. The half-times were 56.6 s, 33.9 s and 24.2 s, respectively, with a release amplitude of 79 ± 1%. (B) Temperature dependence of the displacement reaction of SAV-BcO by unlabeled B7 for the AB7 filling model (at 20°C and 27°C) and for the AB7 model (at 20°C). The corresponding kDISPLACEMENT (calculated from the slope) were 1.6 ± 0.4 × 10² M⁻¹ s⁻¹, 4.6 ± 0.3 × 10² M⁻¹ s⁻¹ and 1.2 ± 0.3 × 10³ M⁻¹ s⁻¹, respectively. (C) Displacement reaction of unlabeled B7 and SAV-BF1 complex (AB7 filling model) at 30°C. The concentration of challenging B7 was 1400 nM which produced a release of only 5% of the bound probe. The green curve is the observed data and black curve is the fitted curve for which only 6.5% displacement was observed for SAV-BF1 complex in contrast with 79% in case of the complex formed with the longer linker BcO.

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**Fig 9. Dissociation kinetics of SAV-BcO and SAV-BF1 complexes.** (A) Concentration dependence of the displacement reaction of SAV-BcO complex (AB7 model) by unlabeled B7 at 20°C. The concentration of challenging B7 was 740 nM (blue), 1240 nM (pink) and 1740 nM (red) after the remaining free binding sites were filled. The half-times were 56.6 s, 33.9 s and 24.2 s, respectively, with a release amplitude of 79 ± 1%. (B) Temperature dependence of the displacement reaction of SAV-BcO by unlabeled B7 for the AB7 filling model (at 20°C and 27°C) and for the AB7 model (at 20°C). The corresponding kDISPLACEMENT (calculated from the slope) were 1.6 ± 0.4 × 10² M⁻¹ s⁻¹, 4.6 ± 0.3 × 10² M⁻¹ s⁻¹ and 1.2 ± 0.3 × 10³ M⁻¹ s⁻¹, respectively. (C) Displacement reaction of unlabeled B7 and SAV-BF1 complex (AB7 filling model) at 30°C. The concentration of challenging B7 was 1400 nM which produced a release of only 5% of the bound probe. The green curve is the observed data and black curve is the fitted curve for which only 6.5% displacement was observed for SAV-BF1 complex in contrast with 79% in case of the complex formed with the longer linker BcO.

**Reaction model of biotin-DNA ds-F1 binding to AV and SAV.** The B7 binding kinetics, when attached to DNA, was best described by two parallel reactions (Eq 9) with two independent association rate constants that showed no evidence of intermediates in solution. The pre-exponentials of the rate constants were temperature dependent (Table 2) suggesting the presence of two B7 populations with different orientations with respect to the DNA and responsible for the measured kon1 and kon2 rate constants. Thus, at 25°C, the measured values of kon1 for both AV and SAV were only 20–40% slower than rate constants acquired with BFl, which suggests that B7 on the DNA was positioned in a favorable orientation that enhances the association reaction. On the other hand, the slower kon2 rate constant is associated with an unfavorable orientation of the second B7 population which could be partially intercalated in the stacked nucleotides.

**Thermodynamic parameters**

The forward activation energies (Ea^forward or ΔH^forward) of the B7 binding to AV and SAV were ~6.0 and ~14 kcal/mol, respectively; and they were in good agreement with early estimations of 10–12 kcal/mol for the displacement of water molecules from the binding pocket [60]. These values were larger than the 3–4 kcal/mol [35, 73] characteristic of a diffusion limited reaction (which requires also association rate constants in the order of 10⁹ M⁻¹ s⁻¹ and ~7.5 × 10⁶ M⁻¹ s⁻¹ at 25°C for AV-BFl and SAV-BFl, respectively). Hence, the association reaction is not diffusion controlled in the range of experimental work carried by us. Interestingly, the B7 binding process for both proteins share the same kon at 52.1°C (calculated by Arrhenius plot), and binding of B7 ligand enhances thermal stability of the proteins shifting from 75°C to 112°C for SAV and from 84°C to 117°C for AV [74].

Remarkably, the difference of forward and reverse activation energies (Ea^forward — Ea^backward), calculated with Arrhenius plots of the association and dissociation rate constants, respectively; matched, within the error, the reaction enthalpy (ΔH^Rxn) calculated by calorimetry (Table 3, Fig 10A). The same argument holds for the Gibbs free energy (ΔG^#, forward—ΔG^#, backwards) and entropy (ΔS^#, forward—ΔS^#, backwards), and the calorimetric ΔG^Rxn and ΔS^Rxn values have been
Table 3. Thermodynamic cycles of B$_2$ binding to AV and SAV for one transition state.

| Complex | 1) $\Delta H^\circ_{\text{rxn}}$ (kcal/mol) | 2) $\Delta S^\circ_{\text{rxn}}$ (cal/mol K) | 3) $E_a$ Forward, a (kcal/mol) | 4) $E_a$ Backward, b (kcal/mol) | 5) $\Delta E = E_a$ Forward $- E_a$ Backward (kcal/mol) column 3 minus 4 |
|---------|----------------------------------------|---------------------------------------|--------------------------------|--------------------------------|-------------------------------------------------|
| AV-B$_2$ | -20.3 ± 0.3 [76] | -23.4 ± 0.3 [78] | 14.4 ± 0.2 | 37.6 ± 2.0 [18] | -23.2 ± 2.2 |
| SAV-B$_2$ | -23.0 [79] | -24.5 [39] | 6.0 ± 0.2 | 32.0 [81] | -23.4 ± 4.0 |

| Complex | 1) $\Delta S^\circ_{\text{rxn}}$ (cal/mol K) | 2) $\Delta G^\circ_{\text{rxn}}$ (kcal/mol) | 3) $\Delta G^\circ_{\text{rxn}}$ Forward (kcal/mol) | 4) $\Delta G^\circ_{\text{rxn}}$ Backward (kcal/mol) | 5) $\Delta G^\circ_{\text{rxn}}$ Forward $- \Delta G^\circ_{\text{rxn}}$ Backward (kcal/mol) column 3 minus 4 |
|---------|----------------------------------------|---------------------------------------|--------------------------------|--------------------------------|-------------------------------------------------|
| AV-B$_2$ | -8.9 [78] | -20.5 [76] | 4.7 ± 0.3 | 24.7 ± 2.0 [18]$^c$ | -20.0 ± 4.0 |
| SAV-B$_2$ | -21.0 [39] | -18.1 ± 0.3 [83, 84] | 4.0 ± 0.6 | 21.4 ± 2.0 [47] | -19.6 ± 2.5 |

Experimental forward parameters ($E_a$ Forward, $\Delta S^\circ_{\text{Forward}}$, and $\Delta G^\circ_{\text{Forward}}$, in column 3) calculated by us, are in a good agreement with the experimental calorimetry values ($\Delta H^\circ_{\text{rxn}}$, $\Delta S^\circ_{\text{rxn}}$ and $\Delta G^\circ_{\text{rxn}}$, in column 2) and dissociation parameters ($E_a$ Backward, $\Delta S^\circ_{\text{Backward}}$, and $\Delta G^\circ_{\text{Backward}}$, in column 4) when a one-transition state reaction model is considered.

$^a$Column 2 and 4 contain data obtained in previous studies. Comparisons between reported values are made, and the average value is placed below the dotted line.

$^b$The forward and reverse rate constants were used to calculate, with Arrhenius plots, the respective forward and backward activation energies, $E_a$ Forward and $E_a$ Backward, respectively.

$^c$Column 5 is column 3 minus column 4 and should be equivalent to experimental reaction values obtained from multiple studies thus confirming the accuracy of the proposed model. The difference of these activation energies results in $\Delta E$ (column 5) which were equivalent to an averaged $\Delta H^\circ_{\text{rxn}}$ (column 1) of multiple independent calorimetry studies. Similarly, analysis was carried out for reaction Gibbs free energy ($\Delta G^\circ_{\text{rxn}}$) and entropy ($\Delta S^\circ_{\text{rxn}}$).

$^d$Averaged values are shown below the dotted line.

$^e$Calculated from the plotted data.

$^f$Calculated at 25°C.

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calculated by others (see references in Table 3, Fig 10B and 10C); Thus, the forward thermodynamic parameters obtained in this study completed nicely the thermodynamics cycles, thus making very compelling arguments in favor of the proposed simple reaction model (Eq 8), which has a single transition state (5) but no intermediate. The positive nature of $\Delta G^\circ_{\text{Forward}}$ and $\Delta S^\circ_{\text{Forward}}$ toward the transition state can be explained as the energy required to remove water molecules and displace the protein’s β3-β4 loop [32, 75] with an increment of the overall
Table 4. Spectroscopic parameters of the dye-labeled B2 probes and respective complexes with AV and SAV.

| Sample a | Maximum Absorbance (nm) | Maximum Emission (nm) | Lifetime τ b | Natural Lifetime τ c-1 | Dynamic Quantum Yld Φ d | Quantum Yld QY | Fraction of Non-Statically Quenched Dye 1-S | Steady State Anisotropy r ss e | Cone Angle Ω f |
|----------------|------------------------|-----------------------|--------------|------------------------|------------------------|-------------|--------------------------------|----------------|---------------|
| BFI           | 494                    | 530                   | 4.03 ± 0.01  | 5.2 ± 0.2              | 0.81 ± 0.04            | 0.52 ± 0.02 | 0.65 ± 0.06                     | 0.021 ± 0.002   | 90            |
| AV-BFI        | 498                    | 528                   | 4.22 ± 0.01  | 4.7 ± 0.1              | 0.90 ± 0.02            | 0.44 ± 0.01 | 0.49 ± 0.03                     | 0.180 ± 0.003   | 51 ± 2        |
| SAV-BFI mono-ionic | 472              | 515                   | 3.0 ± 0.1    | 8.1 ± 0.1              | -                      | 0.06 ± 0.011 | 0.18 ± 0.02                     | 0.171 ± 0.008   | 51 ± 2        |
| SAV-BFI di-ionic | 494                 | 528                   | 4.1 ± 0.1    | 4.5 ± 0.1              | -                      | 0.49 ± 0.03 | 0.42 ± 0.03                     | 0.09 ± 0.002    | 50 ± 2        |
| BcO           | 495                    | 523                   | 3.75 ± 0.04  | 4.4 ± 0.1              | 0.85 ± 0.02            | 0.91 ± 0.02 | 1.00 ± 0.02                     | 0.023 ± 0.002   | 90            |
| AV-BcO        | 497                    | 524                   | 4.35 ± 0.01  | 4.9 ± 0.1              | 0.88 ± 0.02            | 0.62 ± 0.02 | 0.70 ± 0.03                     | 0.187 ± 0.009   | 50 ± 2        |
| SAV-BcO       | 497                    | 524                   | 3.98 ± 0.01  | 4.8 ± 0.1              | 0.83 ± 0.02            | 0.21 ± 0.01 | 0.25 ± 0.02                     | 0.053 ± 0.005   | 50 ± 2        |
| B2-DNA ds Fl  | 502                    | 520                   | 3.12 ± 0.08  | 4.3 ± 0.1              | 0.75 ± 0.04            | 0.22 ± 0.01 | 0.29 ± 0.02                     | 0.077 ± 0.005   | 49 ± 2        |
| AV-B2 -DNA ds Fl | 499              | 521                   | 3.80 ± 0.05  | 3.9 ± 0.1              | 0.98 ± 0.02            | 0.36 ± 0.01 | 0.37 ± 0.02                     | 0.150 ± 0.007   | 40 ± 3        |
| SAV-B2 -DNA ds Fl | 499             | 521                   | 3.86 ± 0.01  | 3.9 ± 0.1              | 1.00 ± 0.02            | 0.18 ± 0.01 | 0.18 ± 0.02                     | 0.082 ± 0.001   | 51 ± 3        |

The chemical environment is altered after complex formation as changes in the following properties shown: shifting in absorbance peaks (abs max, S1 Fig) and fluorescence emission peaks (emi max, S2 Fig), lifetimes (τ), dynamic quenching (Φ), static quantum yields (QY), fluorescence emitting population (1-S) and the cone angle that indicates dye mobility (Ω).

aExperiments were carried out with protein excess (AB2 filling model).
bBi-exponential decays were observed for the B2-DNA ds Fl and SAV-BFI complexes (S1 Table).
cτ, is the intrinsic lifetime of the fluorescent dye when there are no other radiationless transitions.
dΦ = Σtτ/τ c, is the dynamic quantum yield.
e1-S = QY/Φ, is the fraction of non-statically quenched dye.
r ss, is the steady state anisotropy measured at 20°C.
fΩ, is the cone angle measured in degrees at 20°C.

The absorbance spectrum of the SAV-BFI complex (S1 Fig) and the detection of the corresponding lifetimes of 3.0 and 4.1 ns [85, 86] indicates the presence of both Fl1- and Fl2-, respectively [79]. We used these reported lifetimes to calculate the pre-exponential values (α) of each fluorescent species in the SAV-BFI complex. The intrinsic lifetime of Fl1- was calculated by dividing the lifetime (3.0 ns) over the absolute quantum yield (0.37) [46]. We calculated the (1-S) τ Fl by assuming that (1-S) τ Fl 2- is that of AV-BFI (with contains only Fl2-) and solving the following equation: \( \frac{1}{\tau} = \frac{1}{\tau_1} \frac{\alpha_1}{1 + \frac{\alpha_1}{\alpha_2}} \), where C is the concentration, ε is the molar absorptivity at 470 nm, D is the fraction of photons under a band width of 520 nm ± 5 nm of the normalized emission spectrum of Fl1- and Fl2- (taken from Fig 7 [86]) which values were 0.229 and 0.158, respectively. The α2 and α1 were 0.611 and 0.388, respectively; for a ratio of 1.574. The concentration of Fl2- and Fl1- were acquired by solving simultaneously the following equations: \( \text{abs} = (\varepsilon^{494} \cdot C)^{1-} + (\varepsilon^{494} \cdot C)^{2-} \) and \( \text{abs} = (\varepsilon^{472} \cdot C)^{1-} + (\varepsilon^{472} \cdot C)^{2-} \), where abs is the absorbance at 494 nm and 472 nm of the SAV-BFI absorbance spectrum; and the ε of Fl1- is 25 mM-1 cm-1 and 29 mM-1 cm-1 at 494 nm and 472 nm, respectively; and ε of Fl2- were 76 mM-1 cm-1 and 35 mM-1 cm-1 [86, 87] at 494 nm and 472 nm. Thus, for the SAV-BFI complex, the concentration ratio of Fl2-/Fl1- was 3.5.

The QY and Φ of the SAV-BFI complex could not be resolved for each of the two Fl species.

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Disorder, ΔS‡. A comparative analysis of the transition state (T) for the AV-B and SAV-B complexes reveals that the former has a larger ΔS‡ forward and ΔS‡ forward (Table 3, Fig 10, red line) than the latter (Table 3, Fig 10, green line) which implies that binding sites of AV are deeper and less accessible resulting in slower association rate constants and larger activation energy with respect to B2 binding to SAV.

Fluorescence spectroscopic parameters of labeled probes and complexes

The absorbance and emission peaks of all the dye-labeled B2 complexes (Table 4) were red shifted a few nanometers (Supporting Information, S1 and S2 Figs) with respect to the
In the presented study, we calculated the association rate constants of B
unbound probes, with the exception of the B
dependent association and dissociation rate constants were used to calculate the E
changes. Thus, in the case of the B
model (Eq 9) with two reacting populations: (Fl) whose exponentials were not affected by temperature suggesting the existence of the two Fl positions on the DNA which make a compelling argument for the parallel reaction model (Eq 9) with two reacting populations: (Biotin-DNA
Fl complexes of SAV and AV proteins (S1 Table) had two lifetimes decays of 0.72 (± 0.01) ns and 3.78 (± 0.01) ns, and 2.29 (± 0.02) ns and 4.08 (± 0.01) ns, respectively; whose exponentials were not affected by temperature suggesting the existence of the two Fl positions on the DNA which make a compelling argument for the parallel reaction model (Eq 9) with two reacting populations: (Biotin-DNA
Fl was QY = 0.22
s
binding to AV and SAV. (A) Enthalpy, (B) entropy, and (C) Gibbs free energy of the B7 binding to AV (red) and SAV (green) for one transition state and no intermediate. The ΔH
rxn
, ΔS
rxn
, and ΔG
rxn
correspond to the average values found in multiple studies (Table 3). Arrhenius plots of the temperature dependent association and dissociation rate constants were used to calculate the E
forward and E
backwards, respectively.

Conclusions
In the presented study, we calculated the association rate constants of B7 binding to AV and SAV with dye-labeled B7 probes and unlabeled B7. We concluded that attached fluorescent probes did not alter the association rates and no binding cooperativity was observed when comparing the initial (unoccupied) and final (occupied) binding rates. The fluorescence, \( F(t) \), and corrected anisotropy signals, \( F'(t) \), of the dye-labeled B7 probes provided truthful binding traces contrary to the uncorrected anisotropy signal, \( \tau(t) \), due to changes in the QY of the participating reacting species. The B7 association rate constants of SAV are several times faster than AV and the glycan chain of the latter does not play a role in the B7 binding association and neither explains the difference in the \( k_{on} \) values between these two proteins. Thus, we conclude that the main differences in reaction speeds are likely related to the accessibility to the binding pocket in solution, and due to the open form in the shorter loop in SAV (residue 45 to 52, 8 residues) [81] in comparison with the AV’s 12-residue loop L2-L3 (residue 35–46) [89].

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Detailed characterization of avidin, strepavidin, and ligands
Also, the variation in requirements for an induced fit could explain larger activation energy and entropic increment for AV compared to the SAV in the overall thermodynamics of the reaction. Interestingly, the overall reaction free energy changes are equivalent.

The association rate constant for BcO, in which the tag is attached to a longer linker of biocytin, is ~2X faster than B7 with the shorter linker (BFl) for both proteins. The difference of 100X in K\textsubscript{D} of AV complex with B7 and biocytin can be explained by differences in the dissociation process rather than the association rate constants. The B7 binding to AV and SAV is not diffusion limited as larger than 3 kcal/mol activation energies were calculated with Arrhenius plots of the rate constants, and those rates were two orders of magnitude slower (on average ~10\textsuperscript{7} M\textsuperscript{-1}s\textsuperscript{-1}) than the 10\textsuperscript{9} M\textsuperscript{-1}s\textsuperscript{-1} required for diffusion limited reactions. The forward thermodynamic parameters of B7 binding to AV and SAV complemented nicely the thermodynamic cycles with data obtained with independent calorimetric studies and dissociation kinetics elsewhere. Thus, the most probable reaction model is the one without a chemical intermediate and a single transition state in solution, but it could be more elaborate on support matrices, such as in chip assays.

The spectroscopic properties indicated very compact complexes with high dye mobility for all the probes, BFl, BcO and B\textsubscript{7}-DNA\textsubscript{ds}\textsuperscript{"Fl}. We report for the first time a bimolecular displacement rate constant value for the SAV-BcO complex when challenged by unlabeled B7 and this displacement of the B7 with the longer linker (biocytin) in the BcO; this suggests that the repair and reconditioning of enriched B\textsubscript{7}-avidin-like surfaces is possible if long linkers are used. Early observations of affinity variations depending on the linker lengths for similar dye-labeled B\textsubscript{7} probes have been showed in incubation anisotropy titrations [90] but the paper did not systematically study the rate constants at various conditions (Tables 1 and 2) and multiple spectroscopic values (Table 4) of the probes as carried out here.

The AV and SAV complexes are highly thermally stable at 112°C and 117°C [74]; respectively, and a possible application of dye-labeled B\textsubscript{7} and AV-like complex could be in Dye- Sensitized Solar Cells (DSSC) [91, 92] as the photon harvesting dye can be displaced when damaged. The protein can be attached covalently to the n-type material (e.g. TiO\textsubscript{2}) and the charge-transfer
molecule to B7 (e.g., porphyrins, chlorophylls, ruthenium-complexes, coumarins or indoline dyes [93]), with the advantage of regeneration capabilities, as damaged dye can be reconditioned or replaced by another dye type on the tetramer attached surface (Fig 9A). This technique could be simpler than switchable mutants of avidin for regenerative biosensors reported elsewhere [94, 95]. The spectroscopic properties of these dye-labeled B7 and AV-like complexes are vital for detection methods based on polarization, fluorescence, anisotropy and Fluorescence Resonance Energy Transfer (FRET) systems because static, dynamic quenching and rotational constraints of the fluorescent probes reduce the detection limits by decreasing the signal to noise ratios [96] and producing artifacts. The information here presented will be valuable to improve new nano-technological applications of B7 and AV-like protein systems.

Supporting information

S1 Fig. Absorbance spectra of dye-labeled B7 probes and respective complexes with AV and SAV. The absorbance spectra of the unbound BFl, BcO and B7-DNA_{ds}^{+}Fl are shown in dark blue and the respective bound complexes formed with SAV and AV in pink. The spectra are normalized to the calculated molar absorptivities. The distortion of the SAV-BFl absorbance spectrum (B panel) is caused by the presence of Fl^{2-} and Fl^{1-}. The labeled probes and protein concentrations were 1 μM and 10 μM, respectively. (DOCX)

S2 Fig. Fluorescence emission spectra of dye-labeled B7 and respective complexes with AV and SAV. The normalized spectra of the unbound BFl, BcO and B7-DNA_{ds}^{+}Fl are shown in blue and their respective bound complexes formed with SAV and AV in pink. The probe and protein concentrations were 20 nM and 1040 nM (AB1 filling model), respectively. (DOCX)

S3 Fig. Dissociation reactions of AV-BcO and AV-BFl complexes by unlabeled B7 at 20˚C. The dissociation reactions of AV complexes were carried out with a preformed complex of 20 nM BFl or BcO and 260 nM AV for a filling model of AB1 and challenged with unlabeled B7 at 2,000 nM. The k_{displacement} could not be detected and the corresponding k_{replacement} (9 x 10^{-8} s^{-1}) found by Green N. [35] is too slow to be determined by the our fluorescence anisotropy methodology. (DOCX)

S1 Table. Lifetimes of dye-labeled B7 probes and protein complexes. The fluorescence lifetimes are shown in nanoseconds and were obtained in solution. (DOCX)

S1 File. Excel file with data values. (XLSX)

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