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# Avidin cooperative allosterism upon binding biotin observed by differential changes in intrinsic fluorescence

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A B S T R A C T

Similar to streptavidin, the binding of biotin by avidin does not appear to be cooperative in the traditional sense of altered binding strength, though it appears to be cooperative in terms of ligand induced structural communication across subunits in the protein as previously shown for streptavidin. In this work we provide data from intrinsic tryptophan fluorescence as evidence of a cooperative structural change. The technique involves examination of the changes in fluorescence emission corresponding to the various tryptophan populations accompanying avidin-biotin binding. We note that the 335 nm emission population (i.e. more hydrophobic local environment) saturates prior to full ligation and the saturation of the 350 nm emission population commonly used in standard binding activity assays. We also note that total integrated fluorescence emission and peak height during the titration of ligand into streptavidin also reach saturation prior to the 4:1 stoichiometric end point. Unique to avidin and distinct from the behavior of streptavidin described in our prior work, the wavelength of maximum emission and full width at half maximum (FWHM) data do not saturate prior to the 4:1 stoichiometric end point. Avidin also exhibited larger FWHM for both apo and holo forms suggesting greater heterogeneity in local tryptophan environments, as compared to streptavidin. Taken together, the data suggests that the binding of the first 3 biotins effect greater structural changes in the protein than the final ligand in a similar way for avidin and streptavidin.

## 1. Introduction

The specific binding of biotin (vitamin H) to avidin and the analogous soil bacterium protein, streptavidin, has an affinity that is essentially stoichiometric. Due to this specific and high affinity interaction ($K_d \approx 10^{-15}$), these proteins have been the focus of much study and have been used in the development of a wide range of biotechnology and biomedical techniques.

While quite similar in terms of the strong and specific binding of biotin, avidin and streptavidin have some significant differences in their structure. One of these differences is that avidin is glycosylated, while streptavidin has no associated sugars. Additionally, streptavidin contains 6 tryptophan residues per subunit, avidin only contains 4 tryptophan residues, which serve as structural probes of the protein conformation via intrinsic fluorescence. In streptavidin 4 of the tryptophan residues are within the binding site, while in avidin there are only 3. Upon excitation at 280 nm, the tryptophans in both avidin and

streptavidin exhibit an emission maximum at 333–336 nm, indicating that they are relatively inaccessible to solvent (i.e. a more hydrophobic environment) [1–3]. Intrinsic tryptophan fluorescence emission occurs at longer wavelengths in when the residues are in more hydrophilic environments. This makes tryptophan emission an indicator of local chemical environment [3].

In prior work with streptavidin, saturation with biotin caused a fluorescence blue shift of 4–8 nm accompanied by a 25–39% decrease in emission intensity at 350 nm [1,4]. Others have shown that monitoring fluorescence intensity at 350 nm as a function of biotin added provides a reliable assay for the stoichiometric binding of biotin to (strept)avidin [5,6].

Both tetrameric proteins are known to be quite stable with respect to heat with a $T_m$ greater than 75 °C in the absence of denaturant, and over 100 °C when saturated with biotin [7,8]. Of particular note, the quaternary and tertiary structure of streptavidin has also been shown to be quite stable even in the presence of common denaturants (i.e. SDS and

urea) [8,9]. For example, the activation energy of unfolding of SA is still over 100 kJ/mol in the presence of 1.0 % SDS [8].

Traditional definitions of cooperativity involve multiple binding sites where the binding of a ligand to the first site increases the affinity of subsequent ligands as measured by equilibrium binding constants. In our prior work we proposed the concept of cooperative allosterism to describe situations where ligand binding induces structural rearrangements across subunits without changing the intrinsic binding affinity, as in binding of biotin to streptavidin [10]. The simplest way to describe this cooperative allosteric phenomenon is to view the tetramer of avidin as a dimer of dimers. Upon ligation of one subunit, allosteric effects are transduced to the opposing monomer of that dimer which alters the fluorescence properties of both the ligated subunit as well as the opposing half of the dimer, perhaps by a lesser amount (e.g. Trp-120 in streptavidin or Trp-110 in avidin). Upon binding a second ligand, statistically favoring the unligated dimer since it has two open binding sites. This would result in a situation similar to the first ligation event. Therefore, the subsequent third and fourth ligations would elicit a lesser effect than the first ones. Because this effect is only apparent at 335 nm emission and lower, it would indicate that the more solvent exposed tryptophans (seen at 350 nm) are not affected in the same way as the more buried tryptophans. This work examines the similarities and differences observed for avidin binding to biotin, as indicated by the intrinsic tryptophan fluorescence changes.

## 2. Materials & methods

**Materials** Hen Egg White Avidin was obtained from EMD Millipore Corp. (Burlington, MA) and used without further purification (catalog # 189725, Lot # 3222635, 95.0% AV in lyophilizate, 14.8 U/mg protein activity is 98.7 % active binding sites). Protein stock solutions were prepared at roughly 3–8 mg/mL in 10 mM triethanolamine (TEA) at a pH 7.3 (titrated with HCl). Avidin concentration was determined using $A_{280}$ with $\varepsilon^{1\ mg/mL}_{280} = 1.66$ cm$^{-1}$. Type I, 18 MΩ water was used to prepare all solutions. D-biotin was obtained from Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO) and also dissolved in the 10 mM TEA buffer at pH 7.3.

**Fluorescence spectroscopy** A Photon Technology International (PTI, Lawrenceville, NJ) QuantaMaster Dual-Emission Spectrofluorimeter with a thermostatted single sample cell was used for all fluorescence analyses. Excitation illumination at 280–290 nm was provided by a 75 W output power Xenon arc lamp. Bandpasses were 2 nm and 8 nm for excitation and emission, respectively. Photomultiplier tube (PMT) voltages were 980–1000 V. Upon each addition of protein or ligand to the solution, a 2 min equilibration period was allowed. Samples were subjected to constant stirring.

In all cases the excitation shutter was closed between readings to avoid photobleaching. In timebased experiments where multiple emission wavelengths were examined, all emission wavelengths were examined on each instrument channel. Fluorescence emission intensity was corrected for background (i.e. buffer as blank) emission and dilution of streptavidin.

A nominal Avidin concentration of 0.9–1 μM provided emission maxima of approximately 1.9–2.8 × 10$^6$ counts/s, well under the 3.7 × 10$^6$ signal saturation point of the instrument. A standard 1 cm path length quartz cuvette was used for all experiments. Typical experiments began with a 2 mL volume of protein solution then titrating in 5–20 μL aliquots of 38–100 μM biotin solution. Concentration of biotin solutions was determined using the standard 350 nm emission titration curve [5, 6].

## 3. Results

As shown in Fig. 1, the intrinsic tryptophan fluorescence emission intensity undergoes a significant blue-shift, as well as a quenching. The
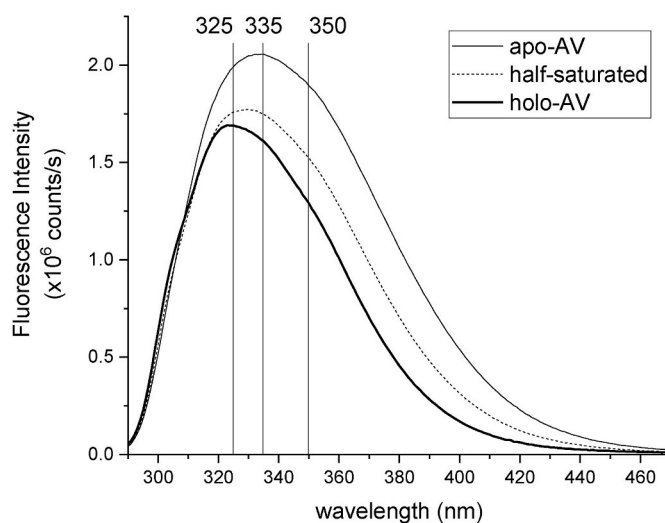


**Fig. 1.** Fluorescence emission spectra of avidin at varying levels of biotin saturation (as indicated).

emission $\lambda_{max}$ decreases from 335 nm to 325 nm (±1.5 nm) going from the apo-to the holo-avidin form. This blueshift of 10 ± 1.5 nm is somewhat larger than the 7–8 nm shift found previously for streptavidin. Despite this blue-shift in the emission upon binding, one can clearly still see a significant shoulder above 350 nm owing to less perturbed Trp residues beyond the binding site. The intensity of the emission decreases as tryptophan fluorescence is quenched due to interactions between 3 of the Trp residues with biotin in the binding pocket. In total intensity, the fluorescence emission decreased by 33 ± 8% going from apo-to holo-avidin, which is similar to the 30% decrease found previously for streptavidin [10].

In order to examine the potential influence of tyrosine fluorescence, we also performed emission scans for samples excited at 290 nm. When the data are normalized to apo emission at 335 nm, Fig. 2 shows that the fluorescence emission profile is essentially the same for both 280 nm and 290 nm excitation. On the other hand, with the same normalization factor applied, the emission intensity for the 290 nm excitation of holo-avidin is greater than that observed with 280 nm excitation. The blue-shift observed for the 290 nm excitation was also less than observed for the 280 nm excitation (i.e. 335 nm–329 nm rather than 325 nm).
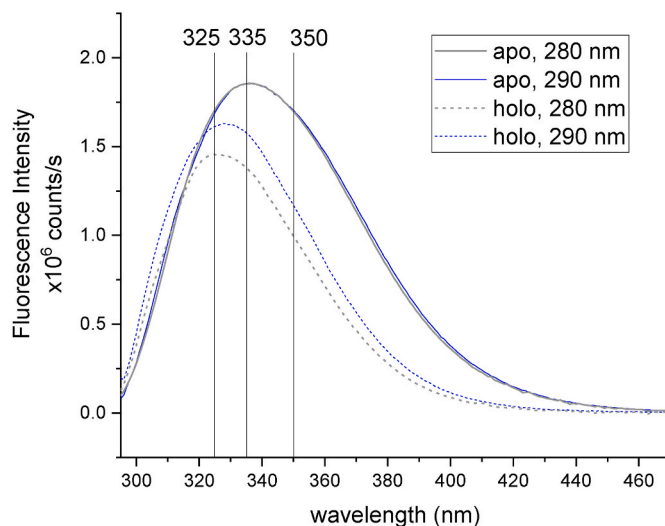


**Fig. 2.** Avidin emission spectra for $\lambda_{ex} = 280$ and 290 nm. Data have been normalized to the maximum emission intensity for the apo form with 280 nm excitation.

Further analysis of the emission spectra shows distinctions between excitation at 280 nm and 290 nm (which minimizes excitation of Tyr [3]). This is summarized in Table 1. The decreases observed for total area (i.e. total fluorescence intensity) are distinct (p = 0.0064), suggesting some contribution of Tyr fluorescence when exciting at 280 nm. However, while the decrease in emission maximum ($\lambda_{max}$) is distinct for the different excitation wavelengths (p = 0.00045), as is the decrease in height (p = 0.037), the decrease in FWHM is not different. This suggests something more complex than just the elimination of shorter wavelength tyrosine fluorescence upon excitation at 290 nm. Li et al. have shown that there can be FRET interactions between Tyr and Trp residues [11].

Similar to what we have previously reported for streptavidin, we performed a series of titrations of biotin into avidin while monitoring the emission at two different wavelengths. A representative plot of these data for 350 nm and 335 nm emission are shown in Fig. 3.* Based on prior work using the 350 nm emission signal for binding assays, and the activity of the avidin protein being 14.8 U/mg, we calibrate the break-point for that data to be 4:1 [biotin]:[AV] [10]. So as not to bias the results, we eliminate the point closest to 4:1, as well as the one prior and immediately after, from the linear fits to the data. As was the case for streptavidin, we consistently see that the 335 nm emission data saturate sooner than the stoichiometric 4:1 [biotin]:[AV]. Table 1 summarizes results from these experiments, which includes examining the what happens for excitation at 290 nm vs. 280 nm, as well as what happens if the wavelength of maximum emission for the holo form is monitored.

As shown in Table 2, there is an early saturation of the 335 nm emission. There is statistically no difference between the 335 nm saturation point when excited at 280 nm vs. 290 nm (p = 0.23). Interestingly, this data is also indistinct from that of streptavidin, as previously reported (335 nm emission crossing at 3.76 ± 0.18) [10].

We also performed titration experiments where we monitored the emission at 325 nm, which is the wavelength of maximum emission for the holo-avidin. We find that this signal saturates at 3.65 ± 0.08. In the case of streptavidin the signal at its wavelength of maximum emission saturated at the same point as the 335 nm emission signal. In the case of avidin we find that this signal saturates statistically sooner (p = 0.0019) than the 335 nm emission signal.

Consistent with our previously reported streptavidin data, we also plotted titration data using various signal data from analysis of emission scans. Table 3 shows the results for wavelength of maximum emission, Area, FWHM and Peak Height. The wavelength of maximum emission and FWHM appear to saturate above 4:1 and the Area and Peak Height appear to saturate sooner. However, the uncertainties on these data tend to be significantly larger than the previously reported data (i.e. 0.2–0.9 vs. 0.08–0.18). In particular the wavelength and FWHM signals have the most uncertainly. This is not unexpected since the wavelength precision for the monochromator is approximately 0.5–1 nm. The plots were also not as linear (i.e. more scattered) as those shown in Fig. 3. We would also



**Fig. 3.** Fluorescence titration of biotin into avidin, monitoring emission at 335 nm and 350 nm ($\lambda_{ex}$ = 280 nm). The three points nearest the 4:1 expected saturation are not included in the linear fits.

**Table 2**
Observed break points in avidin fluorescence emission signals upon addition of biotin. Uncertainties are estimated using the standard deviation.

| | [biotin]:[AV] at saturation $\lambda_{em}$ (nm) | | | |
|---|---|---|---|---|
| $\lambda_{ex}$ (nm) | 335 nm | 350 nm | % diff. | N |
| 280 | 3.81 ± 0.10 | 4.00 | 5 ± 3 | 29 |
| 290 | 3.74 ± 0.18 | 4.00 | 7 ± 5 | 5 |

| | [biotin]:[AV] at saturation $\lambda_{em}$ (nm) | | | |
|---|---|---|---|---|
| $\lambda_{ex}$ (nm) | 325 nm | 350 nm | % diff. | N |
| 280 | 3.65 ± 0.08 | 4.00 | 9 ± 2 | 5 |

**Table 3**
Observed break points in various avidin fluorescence parameters upon titration with biotin aliquots ($\lambda_{ex}$ = 280 nm). Parameters taken directly from fits to emission fluorescence spectra and plotted as a function of ligand to protein concentration ratio. Uncertainties are estimated using the standard deviation (N = 7).

| | $\lambda_{max}$ | Area | FWHM | Height |
|---|---|---|---|---|
| [biotin]:[AV] | 4.6 | 3.8 | 4.4 | 3.3 |
| Std. dev. | 0.9 | 0.3 | 0.6 | 0.2 |

point out that this is not unsurprising given the complexity seen in Figs. 1 and 2 for how the emission of the entire population of fluorophores changes. Therefore, we take the data in Table 3 to indicate that the wavelength and FWHM are effectively saturating near the expected 4:1 [biotin]:[AV] ratio, while the Area and Peak Height appear to saturate prior to the stoichiometric 4:1 (sooner than the FWHM and $\lambda_{max}$ signals).

To examine the potential effect of photobleaching over the course of a titration experiment, particularly for the scan experiments where exposure times are 90 s, we performed a control experiment for apo- and holo-avidin. For these experiments emission scans were performed initially, then after 10 min of 280 nm excitation and again after 10 min in the dark. Each emission scan was 90 s in duration. We found a 4.3 ± 0.4% photobleach for apo and 0.46 ± 0.07% for holo-avidin, respectively. The apo-avidin did not recover its full emission intensity after the 10 min recovery, but the holo-avidin did. For comparison, streptavidin had 3% photobleach for apo and 1.3% for holo. Given that we observe a 33 ± 8% decrease in total emission from apo to holo this level of

**Table 1**
Decreases in key emission parameters for avidin going from apo to holo form.

| | $\lambda_{ex}$ = 280 nm | | $\lambda_{ex}$ = 290 nm | |
|---|---|---|---|---|
| | average | std dev | average | std dev |
| % decrease, area | 33 | 8 | 22 | 4 |
| % decrease, height | 19 | 9 | 9 | 7 |
| decrease, $\lambda_{max}$ (nm) | 10.3 | 1.5 | 6.5 | 1.2 |
| decrease, FWHM (nm) | 12.9 | 1.0 | 11.6 | 1.7 |

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* Note: The vertical error bars due to the uncertainty in emission intensity is such that they would lie within the area of the data markers used in the figure, so they are not shown.
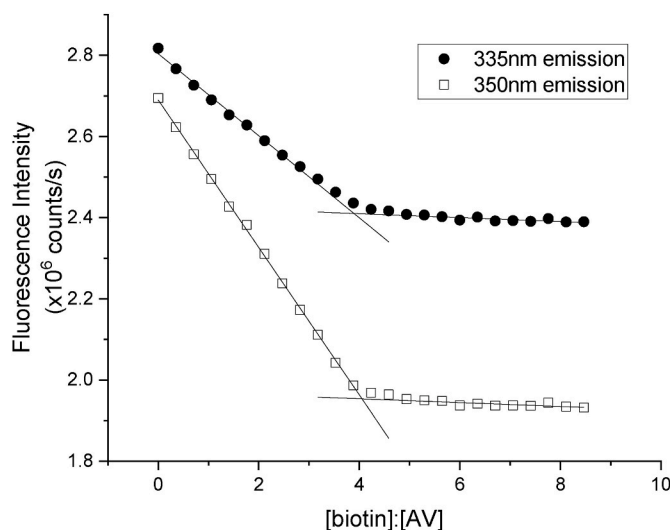
photobleaching after 10 min of constant exposure falls within the uncertainty of the results presented.

## 4. Discussion

The tryptophan fluorescence emission data presented in Fig. 1 ($\lambda_{ex} = 280$ nm) are consistent with prior studies of avidin-biotin binding. We observe a 10 nm blueshift of the fluorescence from apo-to holo-avidin, which is somewhat lower than the 13–14 nm shift reported by Kurzban et al. and Mei et al. [12,13] The $33 \pm 8\%$ quench in emission fluorescence accompanying biotin binding is consistent with the 34% reported by Kurzban et al. [12] The change in FWHM we report, 71 nm (apo) to 58 nm (holo) ($\pm 3$ nm) is larger than the 61 nm–46 nm reported by Kurzban et al. [12] One might note that both Mei et al. and Kurzban et al. used $\lambda_{ex} = 295$ nm.

The shift in $\lambda_{em, \, max}$ from 335 nm to 325 nm ($\pm 1.3$ nm) for apo-to holo-avidin is very close to what we previously reported for streptavidin (335 nm–327 nm) [10]. Similarly, the $33 \pm 8\%$ quench in emission fluorescence for apo-to holo-avidin is consistent with the 30% we have reported for streptavidin [10]. We do note that the FWHM for apo- and holo-avidin, 71 nm and 58 nm ($\pm 3$ nm), respectively, is significantly larger than we found for streptavidin, 57 nm and 50 nm, respectively [10]. This suggests a greater environmental heterogeneity in the tryptophan population for avidin as compared to streptavidin.

Previous x-ray studies of avidin found little difference between the tertiary and quaternary structures of apo and holo forms [14]. However, as is the case for streptavidin, biotin binding does significantly increase the $T_m$ of the protein [4,7,8]. This is attributed to significant secondary structural changes induced by the binding of biotin. In particular, the Trp-120 of streptavidin and the analogous Trp-110 in avidin, which are shifted across the dimer-dimer interface [4,15,16]. Livnah et al. point out that additional hydrophilic and hydrophobic interactions present in the avidin binding site may account for the increased binding affinity as compared to streptavidin [16]. Avidin has 5 aromatic interactions (3 Trp, 2 Phe) and 11 hydrophobic interactions with biotin, which is 1 more aromatic and 3 additional hydrophilic interactions as compared to streptavidin. This is consistent with the observation of an increased FWHM for the tryptophan emission in avidin, since a greater number of Trp interactions in a more diverse range of local environments will lead to a broader emission population. It is also interesting to note that avidin has 75% (3 out of 4) of its tryptophan residues interacting with biotin upon binding, whereas streptavidin has 66 % (4 out of 6) of its tryptophans interacting with biotin. The significant population of tryptophans emitting near 350 nm for holo-avidin suggests that a portion of the tryptophans in avidin remain in hydrophilic environments.

As we showed previously with streptavidin, the fluorescence emission at 335 nm and at the wavelength of maximum emission for holo-avidin saturates significantly before the 4:1 [biotin]:[protein] ratio. The 335 nm emission signal for avidin and streptavidin saturate at statistically the same point ($3.74 \pm 0.18$ and $3.76 \pm 0.18$, respectively). In the case of streptavidin we showed that the emission at the holo-$\lambda_{max}$ saturated at the same [biotin]:[protein] ratio as the 335 nm emission [10]. However, in the case of avidin we find that emission at 325 nm (holo-$\lambda_{max}$) saturates even earlier than the 335 nm emission (p = 0.0019). The result for 325 nm emission data is found at the bottom of Table 2.

In our prior work we made the case for cooperative allosterism in streptavidin using similar analyses of intrinsic tryptophan fluorescence [10]. In that work we make the case that the saturation of tryptophan fluorescence signals prior to [biotin]:[AV] = 4 is consistent with some local chemical environment and structural effects being essentially completed prior to full saturation of the protein with ligand. Recently, Sarter used quasielastic neutron scattering to argue for cooperative molecular dynamics in the streptavidin-biotin system [17]. Again, while it has proven difficult to measure any differences in binding affinity based on level of ligand saturation, owing to the extremely small $K_d$

value, there appear to be differential structural changes, most likely at the secondary structure level, occurring in both avidin and streptavidin that accompany sequential binding events.

While there is similarity between the reported behavior of streptavidin and avidin with respect to the structural changes accompanying biotin binding, as indicated by tryptophan fluorescence, there are some key differences as well. In the case of the fluorescence changes for the avidin-biotin system we have found more complexity in the changes as compared to streptavidin. While both show well behaved (i.e. biphasic linear) plots of $\lambda_{em,max}$, 335 nm and 350 nm emission when excited at 280 nm or 290 nm, plots of other signals vary significantly between the two proteins.

Avidin has only one tyrosine at position 33 which has been shown to be involved in the binding pocket of avidin, while streptavidin has 6 tyrosine residues [18,19]. We found a significant difference in tryptophan emission quench and peak height for excitation at 290 nm vs. 280 nm, with both being less for 290 nm. We also find a smaller blueshift (6.5 $\pm 1.2$ nm) for excitation at 290 nm as compared to 280 nm (10.3 $\pm 1.5$ nm). On the other hand, we do not find a significant difference in the FWHM. Exciting at 290 nm is expected to minimize excitation of the tyrosine residue. Eliminating tyrosine fluorescence would be expected to lower the total fluorescence, but that is not what we have found, which suggests a more complex set of interactions involving the tyrosine and tryptophan residues. This could include FRET interactions between these residues [11]. For example, Gitlin, Bayer and Wilcheck, as well as Livnah et al. have shown that the Tyr-33 interacts directly with biotin in the binding site, in close proximity to Trp-97 [16,18].

Examination of emission intensity (peak area), height, wavelength of maximum emission and FWHM for streptavidin showed consistent linear biphasic curves with behavior similar to the emission intensities at 355 nm and 350 nm. In the case of avidin however, we find greater variability when plotting these signals. As shown in our results, the wavelength of maximum emission and FWHM signals plotted vs. [biotin]:[avidin] saturated above the 4:1 ratio, though with 0.6–0.9 uncertainties.

## 5. Conclusion

We have shown that a number of key tryptophan fluorescence emission parameters are finished changing prior to the 4:1 [biotin]: [avidin] binding point. Significant among these observations are the saturation of 335 nm and 327 nm ($\lambda_{max}$ for holo) as well as total fluorescence emission and peak height. Consistent with our previous study of streptavidin, this behavior is consistent with structural changes of the protein which have finished prior to the binding of the last biotin ligand. As we found for streptavidin, we would propose that avidin also undergoes cooperative allosterism upon binding of biotin.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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