

Experiment 10.1 The Binding of HABA and Biotin to Avidin and BSA.

Background Information: The following information should prove useful in designing your experiment.

Properties of Avidin

Source: egg whites.

Tetrameric (68 kDa); each monomer ~17 kDa.

Binds biotin very tightly: $K_d \sim 10^{-15}$ M.

Each monomer has one binding site; little or no cooperativity.

Spectral Properties

	Max (nm)	ϵ_{282}^*	ϵ_{350}^*	ϵ_{500}^*
Avidin	282	25,000	0	0
HABA	350	2800	20,500	600
Avidin-HABA	500	-----	2000	34,500

*Extinction coefficients are expressed in terms of $M^{-1} \text{ cm}^{-1}$ for either receptor (ϵ_{282}), the free HABA ligand (ϵ_{282} , ϵ_{350} , ϵ_{500}) or bound HABA or the Avidin-HABA complex (ϵ_{350} , ϵ_{500}).

General procedure: We will determine the K_d for HABA and biotin bound to egg white avidin. In order to monitor this reaction, we will use the changes in spectral properties of HABA (shift in wavelength of maximum absorbance and change in extinction coefficient (see Spectral Data above)) upon binding to avidin.

Special Note: This experiment demands a high level of accuracy and precision in your volumetric techniques.

Specifics:

1. Prior to coming to class you should prepare a table that has columns for the following information: vol. of HABA added, total volume, $[\text{HABA}]_{\text{total}}$, A_{500} , $[\text{HABA-Avidin}]$, and $[\text{HABA}]_{\text{free}}$.
2. Each group will be given a stock solution of 36.8 μM avidin in the tetrameric form, from which you can calculate the $[\text{Receptor}]$ (see Table above). Each

group will then prepare a 1 mL avidin solution at a specific concentration. In order to determine the concentration that you need to prepare, consider the following. First, most biological K_d values fall in the range of $< 10 \mu\text{M}$. Second, you want to be able to reach a $[\text{HABA}]_{\text{free}}$ that is $\sim 8K_d$, by titrating in a concentrated solution of HABA (1.07 mM). Third, at the end of the titration, you want a total volume that is less than or equal to 10% greater than your starting volume (WHY?). Based on all of these criteria, I would choose an initial [avidin] in the range of 4 – 6 μM .

3. In order to verify that the correct concentration has been prepared, a spectrum of the solution from 250 nm to 350 nm will be obtained. The concentration will be calculated using the extinction coefficient in the above table.

4. When you are ready to begin collecting your titration data a second baseline will be obtained from 300 nm to 600 nm. Note: since you are using the protein solution as your baseline, all subsequent scans will produce difference spectra (i.e. sample – protein baseline).

5. Before you begin to titrate your protein solution with a solution of HABA you must first determine what will be your final $[\text{HABA}]_{\text{total}}$ based on the assumptions given in #2. There are many different ways to calculate what volume you should add each time. You can add the same incremental amount each time, which is laborious and will take a long time to get to the final volume you need to add. An alternative method is to use a method of doublings, i.e. you make additions of 1 μl , 1 μl , 2 μl , 5 μl , 10 μl , etc. Following the addition of HABA, obtain a spectrum, and recording the A_{350} and A_{500} values.

6. The titration will be essentially complete, when you observe a very small change in absorbance at 500 nm upon addition of more HABA or until you reach the final volume ($\text{vol}_{\text{init}} + 10\%$). Remember that as your textbook points out, the sample will not be completely titrated, but that is not necessary to calculate K_d .

7. On Thursday, we will determine the K_d value for biotin (Objectives 2 and 3 on p. 274). Based on the experience gained from the HABA experiment, you should be able to completely design your own competitive binding experiment.

Data Analysis:

We will analyze the data by graphical methods. In order to do this, it is necessary to use the A_{500} data to determine the [HABA-Avidin] for each addition of HABA. From this data, it is then necessary to calculate the free [HABA]. In order to make this calculation, it is necessary to take into consideration the TOTAL volume of the reaction solution when using the $c_1v_1 = c_2v_2$ equation.

In order to compare the different graphical methods, construct 3 graphs showing:

1. [HABA-Avidin] vs. [HABA]
2. $1/[\text{HABA-Avidin}]$ vs. $1/[\text{HABA}]$.
3. $[\text{LR}]/[\text{L}]$ vs. [LR].

From each plot, determine K_d . For those with computer graphic programs that can perform linear or non-linear regression analysis, compare the value you obtained for K_d by hand-fitting with that obtained by a computer fit. We will discuss in class the results obtained by these different methods of determining K_d . Because of the importance of data analysis and curve fitting techniques, we will attempt to discuss the approaches one takes in critically evaluating the results obtained by linear and non-linear regression analysis.