Physical Chemistry Laboratory I
CHEM 445
BCN
Binding Constant of Neutral Red with Riboflavin Binding Protein[1,2]
02/06/18

Binding affinities of molecules with proteins are given as dissociation constants for the reactions: the larger the dissociation constant the weaker the binding. In order for binding (or complexation) to be significant, the dissociation constants must themselves be small, 10^{-X}.

These dissociation constants are analogous to weak acid ionization constants that you have studied extensively: the smaller K_A, the weaker the acid, the less dissociation at any concentration, and the greater the binding between protons and the anions. Remember the confusion with pK_A: the larger pK_A, the smaller K_A, and the weaker the acid.

Riboflavin Binding Protein, RFBP, binds strongly to riboflavin and is essential to transport riboflavin, Vitamin B2. The pale yellow color of egg whites (Professor White's demonstrations[3]) is due to riboflavin bound to RFBP. The molecular weight of RFBP for chicken eggs (our source) is ~ 30 kDa, although there is variation in molecular weight and structure, depending on the source.

The tri-cyclic flavin ring of riboflavin (Fig. 1, left) binds non-covalently to a complementary pocket in the binding protein (not shown) with complete quenching of the fluorescence of the vitamin. A surrogate compound, Neutral Red, NR, (Fig. 1, right) also binds to the protein and this non-covalent complex can be monitored by changes in the absorption spectra with increasing concentration of the protein.

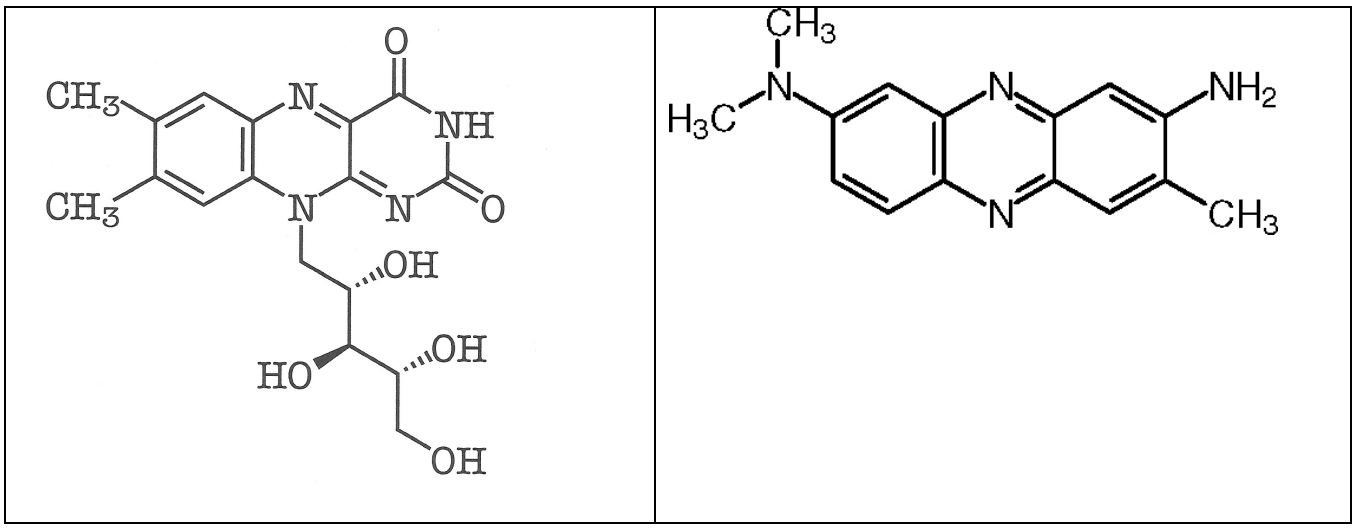

Figure 1. Riboflavin (left) and Neutral Red (right)

Neutral Red absorbs strongly in the visible region and the complex with Riboflavin Binding Protein also absorbs strongly in the visible region, but with a different wavelength for the absorption maximum. The concentrations of all three species at equilibrium are needed to determine the binding constant. In this example, as is often the case in biochemical systems, the concentration of the complex cannot be measured directly – only the total concentrations of the protein, RFBP, and of the ligand, NR. The determination of the binding constant between Neutral Red and Riboflavin Binding Protein will serve as an example of the spectrophotometric technique for determining binding constants.

Experimental Procedure:

You will use stock solutions of accurately known concentrations of Riboflavin Binding Protein, RFBP; of Neutral Red, NR; and a buffer at pH = 9.06. You will mix these to give solutions of different concentrations, measure the absorbance from ~ 350 – 700 nm with the HP 8453 VIS/UV spectrophotometer, and record the absorbance at appropriate wavelengths.

You will be economical with the solutions and add small volumes of the RFBP solution to an accurately known volume of NR, somewhat like a titration, rather than preparing a separate solution for each concentration.

Neutral Red absorbs in the visible region and the pH must be maintained at a constant value because NR is an indicator whose spectrum changes with pH. In addition, pH control is needed because protonated Neutral Red does not bind strongly to RFBF. The concentration of NR must be in the range to allow "reasonable" absorbance values.

The binding protein, RFBP, does not absorb significantly in the 350 – 700 nm spectral region and what is observed in the experiment is a change in the absorption spectrum of NR in the visible region. Consequently, the sequence in the experiment is the addition of RFBP to NR. As you add aliquots of RFBP to the solution, you will note the appearance of a new band in the absorption spectrum of NR at a longer wavelength. This new absorption band is due to the RFBP •NR complex. The increase in the absorbance at the new λ_{max} is proportional to the concentration of the RFBP•NR complex.

The appearance of the new peak in the spectrum for dilute solutions indicates that the formation constant, K_F, for the RFBP•NR complex is large, 10^X, or that the dissociation constant, K_D, is small, 10^{-X}. To do the experiment properly, you should verify the stoichiometry of the interaction. However the complex is known to be 1/1 between NR and RFBP.

I. Use of micropipets

A. Because you may not have used Eppendorf pipets like these, because the technique for preparation of solutions is different from the techniques in QUANT, and to give an indication of your experimental precision, you need to practice delivering an aliquot from the pipet.

It is not necessary to change pipet tips when you are delivering different aliquots of the same solution. It is necessary to use different tips when using different solutions.

You will use a **50 µL pipet** for the aliquots of the protein, RFBP. With a fresh pipet tip, transfer **a 50 µL aliquot** of distilled water into a small vial and weigh on the analytical balance. **{DO NOT ADD WATER TO THE VIAL WHILE IT IS ON THE BALANCE PAN.}** The weighing is easier if you tare the vial before adding water.

Repeat four times to obtain five values. To a reasonable approximation of ± 1 %, you may take the density of water as 1.00 g/mL at room temperature. Calculate the average, standard deviation and 95 % confidence interval of the volume delivered.

Each person will practice using the pipet.

Include these data in your report in tabular form.

If your average value is not ~ 50 µL or if your values show a trend, repeat. The stated volume of 50 µL should be within the 95% confidence interval of your measurements. The pipet is accurate. However, if your value for the volume of the pipet is significantly different from 50 µL, you may use your value for the volume in the calculations.

B. As an additional check on your technique, obtain a Beer's Law plot for Neutral Red at the pH of the buffer solution, ~ 9. A stock solution of NR of known concentration will be prepared for you. Dissolving NR in water takes several hours, even to prepare a dilute solution.

To the accuracy of these experiments the 1000 µL pipet delivers the stated volumes and does not need to be calibrated.

1. Add 2000 µL of buffer at pH 9.06 to the cuvette and use this solution as the blank. {Use the 1000 µL pipet.}

Then add 200 µL of the NR stock solution {using the 1000 µL pipet} to this solution in the cuvette, mix well by inverting the capped cuvette several times {Do not shake: vigorous shaking may introduce air bubbles}, and measure the absorption spectrum of the solution from 350 – 700 nm. Remove the cuvette from the sample holder, invert again a few times, and measure the spectrum a second time. Record absorbance values at λ_{max}{NR}, (453 nm), as 0.XXXX. If the solutions are well mixed, the absorbance values for a given preparation should agree within ± 0.001 absorbance unit, {AU}.

Add another 200 µL aliquot, mix well as before, and measure the absorbance of the solution (twice). Repeat with two additional 200 µL aliquots of NR. You then have four data points. The fit of your data to a Beer's Law plot {A *vs.* C with the intercept = 0} is an indication of the precision of your technique for solution preparation.

The volumes of the added NR stock solution increase the volume of the solution and must be included in the calculation of C_t{NR] in each solution. {C_t{NR} = total concentration of Neutral Red in the solution.} For dilute solutions like these, the approximation that the volume of the final solution is the sum of volumes of the individual solutions is satisfactory.

Although you will not use these data in the analysis, make a Beer's Law plot of Absorbance (vertical axis) vs. C_t{NR]. At the pH of the buffer Neutral Red is in the fully de-protonated form; therefore C_t{NR] = [NR]. The absorbance should be proportional to the concentration of Neutral Red.

2. Repeat the entire procedure. Plot and analyze each set of data. Plot both sets of data in the same figure. The two curves should be nearly superimposable.

Include these data in your report in appropriate tabular form. The plot should be Figure 1. The intercept must be zero: no NR, no absorbance. Use LINEST or Regression to calculate the slope of the curve and the standard error.

If Beer's Law applies to the NR solutions, it should apply to the solutions of the RFBP•NR complex.

II. Determination of the RFBP •NR binding constant

The formation of the RFBP•NR complex is indicated by the appearance of a "shoulder" on the absorption band of NR. You will start with a solution of NR and add small volumes of RFBP solution and measure the absorption spectrum after each addition.

You will be working with small volumes of solutions and a small sample of protein. Be careful. This is your total sample of protein. Pay attention to units. Your concentrations will be in the µM range, $X*10^{-6}$ M.

A. You will have a small Eppendorf tube containing a known amount of the protein, RFBP, MW = 29.2 kDa in the refrigerator. The sample will have your section number and fresh samples will be put out for each experiment. Check to make sure that you are using your sample. The weight will be approximately 10 mg {weighed on a microbalance} and the exact weight will be given. **Add 1000 µL of buffer** to the Eppendorf tube to dissolve the sample. Mix well by repeated inversions of the capped tube, but do not shake to avoid foaming in the cell and the possibility of inactivating the protein.

Put the Eppendorf tube in the constant temperature bath for 15 minutes while completing part **B.**

Calculate the concentration of protein in this (stock) solution using MW{RFBP} = 29.2 kDa {= $29.2*10^3$ g/mole}.

B. Add **2000 µL of buffer** to a clean cuvette and use this as the blank. Then add **1000 µL of the NR stock solution** {1000 µL pipet}, mix well, and measure the absorption spectrum from 350 to 700 nm as before. Record the absorbance **at 550 nm**. This is λ_{max} for the complex, as will become apparent as protein is added.

Add **50 µL** of the RFBP solution, using the 50 µL pipet, to the cuvette containing the buffer and NR, mix well, and record the spectrum from 350 to 700 nm as before..

It is not easy to see the new absorption band of the RFBP•NR complex after the first addition of RFBP. Subsequent additions will show a clear increase in absorption at 550 nm. Record the absorbance at 550 nm to 0.XXXX in your notebook for each solution.

Take the cuvette from the holder, invert a few times, and measure the absorbance again. Record and use both measurements in the analysis.

C. Add another **50 µL aliquot** of the RFBP solution, mix well, measure the absorption spectrum and record the absorbance at 550 nm. Duplicate measurements are needed for each solution. Continue adding the RFBP solution in **50 µL** units to a total of 300 µL and the absorbance has reached a "relatively" constant value.

Calculate the total concentration of NR and of RFBP after each addition. Because of dilution, the total concentration of NR will decrease each time.

D. Repeat a, b, and c for a second determination. Doing the experiment takes little time. You have 1000 µL of solution and used only 300 µL for the first experiment.

Give these data as a table in your report. The primary data should include the cumulative volume of added RFBP, calculated values for C_t{RFBP} and C_t{NR} {including the dilution factor}, A{550 nm}, ΔA{550 nm} = (A{550 nm} at each value of C_t{RFBP}) – (A{550 nm} with no added RFBP, and ΔA{550 nm, corr}, corrected for dilution.

Apply the small correction for dilution of NR. As RFBP is added to the solution, the total concentration of NR decreases and the background subtraction from the NR absorption decreases. Calculate A{550 nm} for the background subtraction by multiplying the original A{550 nm} by the ratio of volumes.

$$\Delta A[550nm, corr\} = A\{550\ nm\} - (A\{550\ nm, w/o\ \ RFBP) \left(\frac{3000\ \mu L}{(3000+V\{RFBP\})\mu L}\right) \quad \{1\}$$

Plot ΔA{corr} vs. C_t{RFBP} in Figure 2.

3. Data Analysis

Calculation of an equilibrium dissociation constant (K_d)

The stoichiometry of the reaction has been independently established,

$$NR + RFBP \Leftrightarrow RFBP\bullet NR \qquad \{2\}$$

One can write the equilibrium expression for this reaction as a formation constant for the reaction as written,

$$K_F = \frac{[RFBP \bullet NR]}{[RFBP][NR]} \qquad \{3\}$$

or as the dissociation constant - the equilibrium expression for the reverse reaction and the general form of the binding constant,

$$K_D = \frac{[NR][RFBP]}{[RFBP \bullet NR]} \qquad \{4\}$$

K_D is often given in units of M, or more often μM or nM: the smaller K_D, the stronger the binding. {Technically, of course, equilibrium constants are properly expressed in terms of activities and have no units.} [RFBP] and [NR] are the concentrations of free RFBP and NR while [RFBP•NR] is the concentration of the bound complex.

Now, the introduction of an experimental accessible parameter, α, which is defined as a ratio of the complex concentration at any concentration of NR versus the total concentration of RFBP ($[RFBP]_0$). In case a ligand (NR) concentration is fixed and a protein (RFBP) is varied, the principle described below can be used to figure out the K_d.

$$\alpha = \frac{[RFBP \bullet NR]}{[RFBP]_0} \qquad \{5\}$$

A change in physical properties such as absorbance (ΔA) upon the formation a complex can be used for determining the K_d if the change in physical properties is directly dependent on the concentration of the complex.

$$\frac{\Delta A}{\Delta A_{max}} = \alpha = \frac{[RFBP \bullet NR]}{[RFBP]_0} \qquad \{6\}$$

Where ΔA is the absorption change and ΔA_{max} is the maximum absorption change produced when RFBP are saturated with NR. α is a fraction of saturation.

Since from Equation {5}, [RFBP•NR] = α∗$[RFBP]_0$ and the concentration of $[RFBP]_0$ is known, thus [RFBP•NR] can be calculated.

Therefore, from Equation {4}, we know that [NR][RFBP] = K_d*[RFBP•NR]. Provide a plot of [NR][RFBP] vs. $[RFBP \bullet NR]$ in Figure 3 and slope will be the K_d.

Learning Goals: Use of spectrophotometer and micropipets; determination of binding constants; data analysis, plotting with Excel; working in pairs; writing technical reports

An Excel table for the data from this experiment is provided on Canvas. Download it as an Excel file. Complete as requested NO CHANGES and NO OMISSIONS and submit to **Canvas site**. Do not include any other data or analysis. This data table is not part of your laboratory report.

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[1] This experiment is based on the article, "Measuring the Binding Affinity of Protein-Ligand Interaction Using Spectrophotometry: Binding of Neutral Red to Riboflavin-Binding Protein", P. Chenprakhon, J. Sucharitakul, B. Panjipan, and P.Chalyen, *J. Chem. Ed*. **2010** *87*, 829.

[2] D. J. Winzor and W. H. Sawyer, Quantitative Characterization of Ligand Binding, Wiley-Liss, New York, 1995, Chap. 3.

[3] D. S. Chatelier and H. B. White, III, *J. Chem. Ed.* **1988** *67*, 814.