JOURNAL OF CHEMICAL EDUCATION

Communication

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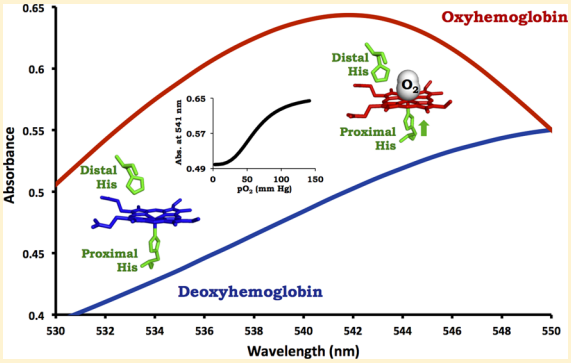
Studying Cooperative Ligand Binding in the Undergraduate Biochemistry Laboratory: Oxygen−Hemoglobin Dissociation Revisited

Megan K. M. Young and Theodore J. Gries*

Department of Chemistry, Beloit College, Beloit, Wisconsin 53511, United States

Supporting Information

ABSTRACT: The interaction between oxygen and hemoglobin is a classic example of a cooperative ligand-binding process. This gaseous-ligand binding process may be studied in the undergraduate laboratory with a variable-pressure cuvette apparatus. An updated method is described to assemble a variable-pressure cuvette apparatus composed of inexpensive supplies and equipment commonly found in the chemistry laboratory.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Laboratory Instruction, Inquiry-Based/Discovery Learning, Biophysical Chemistry, Conformational Analysis, Laboratory Equipment/Apparatus, Proteins/Peptides*

The development of a deep understanding of the effect of cooperativity on ligand binding processes is an important part of undergraduate biochemical and biophysical curricula. Many undergraduate biochemistry textbooks[1−4] and courses use the binding of oxygen to hemoglobin as the quintessential example of a cooperative ligand-binding process. Herein, an updated, easily assembled apparatus is described to study the cooperative nature of the oxygen binding to hemoglobin within the context of whole red blood cells.

Previous undergraduate laboratory activities[5,6] to study the cooperative nature of hemoglobin relied on varying the pressure of the headspace above solutions of blood or hemoglobin to alter the binding state of oxygen to hemoglobin. As the coordination state and local amino acid environment around the heme cofactor changes with oxygen binding, the visual absorption spectrum of hemoglobin changes. Absorbance at a wavelength far from an isosbestic point (i.e., 541 nm) is sufficient to monitor the oxygen saturation of a blood solution.[6] The variable-pressure cuvette apparatus described herein builds upon these previous designs, but eliminates the need for specialized, noncommercial glassware and the determination of the total volume of the system, a process that usually requires mercury. The study of oxygen binding to hemoglobin with this apparatus is suitable to introduce cooperativity in the undergraduate laboratory curriculum.

EXPERIMENTAL PROCEDURE

Defibrinated bovine blood (item no. 828514) was obtained from Carolina Biological (Burlington, NC) and stored at 4 °C. For the experiments described herein, defibrinated blood was used to minimize the time required for preparation of lab materials. Purified hemoglobin may be used, but the Fe^{3+} within the heme cofactors must be reduced to Fe^{2+} and the chosen reducing reagent subsequently removed to observe cooperativity in oxygen binding response.[6] Stopper-top polystyrene disposable cuvettes (item no. A-103) were obtained from Spectrocell, Inc. (Oreland, PA). Clear vinyl tubing with outer diameters of 1.91 and 1.27 cm and 1.905−1.27 cm PEX poly reducing couplings were obtained from Menards, Inc. (Eau Claire, WI). An Agilent UV−visible System 8453 (Santa Clara, CA) was used to record visible-wavelength absorbance data, and a Vernier gas pressure sensor (Beaverton, OR) was used to record the total gas pressure within the apparatus. Two tapered-valve connectors inserted into a No. 5 stopper, a two-way valve, and a 20 mL syringe were supplied with the gas pressure sensor from Vernier, but may be sourced from alternate vendors. A vacuum down to 35 mmHg was drawn using a DuoSeal Vacuum Pump (W. M. Welch Manufacturing Comp., Niles, IL). The total cost to assembly each reusable apparatus was minimal (<$5), if gas pressure sensors, visible spectrophotometers, and magnetic stir plates are available.

Assembly of the Variable-Pressure Cuvette Cell

Approximately 10 cm of vinyl tubing with an outer diameter of 1.91 cm was connected to 30 cm of vinyl tubing with an outer diameter of 1.27 cm with a 1.91−1.27 cm PEX poly reducing

DOI: 10.1021/acs.jchemed.5b00374
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coupling (Figure 1). The larger-diameter tubing end was fitted over the circular top of a stopper-top cuvette containing a

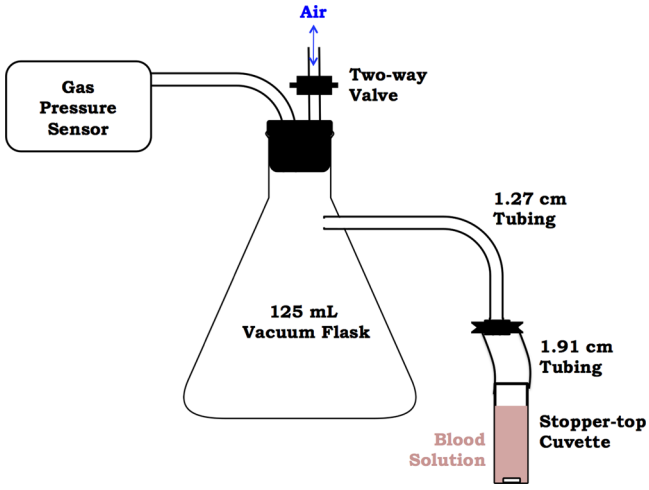

Figure 1. A variable-pressure cuvette cell to study the binding of gaseous ligands to components of solutions within a cuvette.

magnetic stir bar, while the smaller-diameter end was attached to the vacuum port of a standard 125 mL vacuum flask. A No. 5 stopper with two inserted, tapered valve connectors sealed the top of the vacuum flask. A two-way valve was connected to one of the tapered-valve connectors, while a gas pressure sensor was connected to the second tapered-valve connector.

Collection and Analysis of Spectral Data

Bovine blood was diluted 1:200 with 50 mM phosphate buffer, pH 7.4. The pressure within the apparatus was decreased to and stabilized at 35 mmHg using a vacuum pump attached to the two-way value. The blood solution was allowed to equilibrate for 30 min with stirring. The absorbance spectrum was recorded between 520 and 590 nm, and absolute gas pressure within the system was noted. To increase the pressure within the system, a 20 mL syringe filled with 10 mL of air at atmospheric pressure was connected to the two-way value, and the portion of the air was injected into the system. The system was allowed to equilibrate with stirring for 2 min. The absorbance spectrum was recorded between 520 and 590 nm, and absolute gas pressure within the system was noted. The pressure inside the system was incrementally increased, and the absorbance and pressure were recorded until atmospheric pressure was reached.

Absolute pressures of air within the apparatus ($P_{observed}$) were converted to partial oxygen pressures (pO_2) by correcting for the vapor pressure of water (pH_2O; 18.7 mmHg at 21 °C) and considering that oxygen contributes approximately 21% to the total pressure of air (eq 1).

$$pO_2 = 0.21(P_{observed} - pH_2O) \quad (1)$$

The change in absorbance of the blood solution at 541 nm as the pO_2 increased was fit to the Hill equation[7] (eq 2) using nonlinear least-squares curve fitting with Microsoft Excel (Redmond, WA; Supporting Information Figure 1).[8] The absorbance of the deoxygenated blood is represented by the baseline, while the amplitude represents the difference in absorbance between the deoxygenated and oxygenated states. The P_{50} is the pO_2 required for 50% of the binding sites on hemoglobin to be bound by oxygen.

$$A_{541nm} = \text{baseline} + \text{amplitude} \times \left(\frac{(pO_2)^h}{(P_{50})^h + (pO_2)^h} \right) \quad (2)$$

HAZARDS

Handling samples of bovine blood should be performed with appropriate personal protective equipment (e.g., lab coat/apron, gloves, and eye protection). Blood samples and laboratory consumables exposed to blood should be disposed in appropriate biohazardous waste containers.

RESULTS AND DISCUSSION

As the partial pressure of oxygen increases and the hemoglobin within the bovine red bloods cells binds oxygen, the absorbance at 541 nm increases (Figure 2). Interestingly, between 520 and

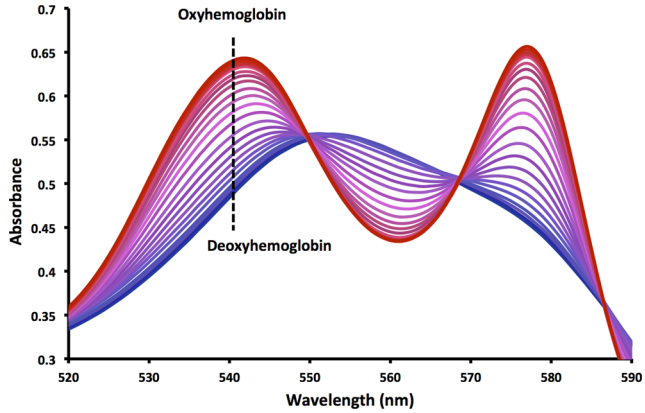

Figure 2. Absorbance of a sample of bovine blood varies significantly between 520 and 590 nm as the partial oxygen pressure is increased from 2.6 mmHg (dark blue curve) to 141 mmHg (bright red curve). The absorbance at 541 nm (dashed line) increases with increasing partial oxygen pressure and is used to monitor the saturation of hemoglobin with oxygen.

590 nm, not all wavelengths have increased absorbance as oxygen is bound. In good agreement with previous research on hemoglobin, isosbestic points at 550, 569, and 587 nm[9] were observed.

For cooperative binding processes, a plot of fractional binding saturation versus ligand concentration should yield a sigmoid-shaped curve. As monitored by the absorbance at 541 nm, the saturation of hemoglobin within the context of defibrinated bovine blood versus the pO_2 generated by the variable-pressure apparatus is shown in Figure 3. The change in absorbance has the characteristic sigmoid shape and is fit well by the Hill equation for a positively cooperative process [h = 3.1 (±0.2), P_{50} = 63 (±1), baseline = 0.499 (±0.002), and 0.157 (±0.004)].

To develop a full laboratory module on ligand binding, the method described herein to study the cooperative nature of oxygen binding to hemoglobin may be combined with the previously described noncooperative process of azide binding to oxidized myoglobin.[10] An example of how to merge these two activities is presented in the Supporting Information. This combined set of activates has been implemented in an upper-division undergraduate biochemistry workshop-format class on protein structure and function that meets three times each week for 2 h of combined class and lab with an enrollment of 15−25 third- and fourth-year students. Working in groups of two or

DOI: 10.1021/acs.jchemed.5b00374
J. Chem. Educ. XXXX, XXX, XXX−XXX

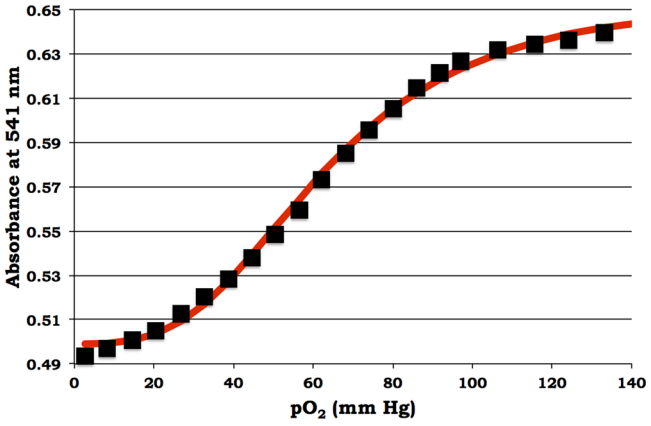

Figure 3. Absorbance of the blood solution at 541 nm (■) increases with a sigmoid shape characteristic of a cooperative ligand-binding process as the partial pressure of oxygen is increased. The data are fit well by the Hill equation for cooperative ligand-binding processes (red curve). The best-fit Hill coefficient is 3.1 (±0.2) with a P_{50} value equal to 63 (±1) mmHg.

three, students completed the entire ligand-binding lab module in as few as 6 contact hours with significant prelab preparation and data analysis outside of class time and in as many as 12 contact hours with all work being completed during class meetings. The updated apparatus presented here is simpler to assemble and more easily manipulated by students, which allows both direct observation of a physiologically relevant cooperative process in the undergraduate lab and time to focus on the interpretation of results. The student success rates were high (≥80%), as long as the equilibration time guidelines were followed. After completing this laboratory module and applicable textbook readings, students were prepared to engage in discussions of other studies involving more complex models for the process of oxygen binding to hemoglobin and the effects of allosteric regulators on the process of oxygen binding to hemoglobin.[11]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00374.

An example Excel worksheet to fit oxygen dissociation data to the Hill equation and a two-part inquiry-based module integrating noncooperative and cooperative ligand binding activities (PDF, DOCX)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: griest@beloit.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to the Beloit College Biomedical Scholars program, the W. M. Keck Foundation, the Mead Witter Foundation, and the Mazur Family and Donald A. Anderson Science Funds for funding.

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DOI: 10.1021/acs.jchemed.5b00374
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