Performing anaerobic stopped-flow spectrophotometry inside of an anaerobic chamber

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Abstract

The catalytic cycle of most flavin-dependent enzymes can be divided into oxidative and reductive half-reactions. Although some enzymes are oxidized by electron carrier proteins or organic compounds, many use oxygen as the final electron acceptor. In order to properly study the reductive half-reaction of flavin-dependent enzyme that react with oxygen, as in the case of oxidases and monooxygenases, it is necessary to establish anaerobic conditions that will only allow the reduction process to be monitored. The reduced flavoenzyme can be further study by expose it to oxygen to monitor the oxidative half-reaction. Anaerobic chambers provide an ideal environment for performing these experiments as they reliably maintain an anaerobic atmosphere in a large workspace. A common tool used to study flavin-dependent enzymes is the stopped-flow spectrophotometry. This chapter describes methods for performing stopped-flow experiments in an anaerobic chamber. We include information about the components of an anaerobic chamber, setting up a stopped-flow spectrophotometer inside of a chamber, preparing anaerobic solutions, and performing experiments to measure the reductive and oxidative half-reactions of flavin-dependent monooxygenases.

Abbreviations

GO	glucose oxidase
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PCD	protocatechuate dioxygenase
SHU	sample handling unit
ITC	isothermal titration calorimetry
UV–vis	ultraviolet-visible

1. INTRODUCTION

Flavoenzymes commonly perform redox chemistry where the catalytic cycle can be divided into reductive and oxidative half-reactions (Neims & Hellerman, 1970). In the reductive half-reaction, the oxidized flavin is reduced by a hydride equivalent to form the reduced flavin (Fig. 1) or via single electron transfer steps where the semiquinone species is formed (Bruice, 1980; Edmondson & Tollin, 1983). During the oxidative half-reaction, the reduced flavin reacts with oxygen, as in the case of flavin oxidases and monooxygenases, or with electron carrier molecules

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Fig. 1. General reaction scheme of the redox cycle of flavoenzymes. The reductive half-reaction is highlighted in yellow and the oxidative half-reaction is highlighted in white. During the reductive half-reaction, the oxidized flavin is reduced to its hydroquinone form by NAD(P)H or other substrates. The reduced flavin then reacts with oxygen and forms various covalent intermediates used for oxygenation reactions or to form hydrogen peroxide.

as in cellular respiration (Dashty, 2013; Romero, Gómez Castellanos, Gadda, Fraaije, & Mattevi, 2018).

Due to the difference in redox potential between oxygen $(E_{O_2+H_2O} = +270 \text{ mV})$ and flavin $(E_{red/ox} = -209 \text{ mV})$, the reaction of these molecules is thermodynamically favorable (Massey, 1994). The reaction begins with a single electron transfer forming superoxide and flavin semiquinone. This radical pair recombines forming either a C4a-per-oxyflavin or C4a-hydroperoxyflavin which are key intermediates in Baeyer-Villiger or hydroxylation reactions, respectively (Fig. 1) (Massey, 2002; Romero et al., 2018). Recently, a flavin-N5 oxide intermediate has also been identified as part of the mechanism of some oxidation reactions (Fig. 1) (Adak & Begley, 2016; Piano, Palfey, & Mattevi, 2017; Teufel et al., 2015).

Recently, research has revealed some insight into how flavoenzymes control the reaction with oxygen. Computational studies combined with site directed mutagenesis have shown that flavin dependent monooxygenases and oxidases use diffusion pathways to direct oxygen to the active site of the enzyme (Baron et al., 2009). In addition, oxygen pressurized X-ray crystallography has shown that the exact orientation of the oxygen can dictate intermediate formation (Saleem-Batcha et al., 2018). It has also been shown that key residues regulate access of molecular oxygen to reduced flavin. These have been named "gate keepers" in studies with vanillyl-alcohol oxidases (Leferink et al., 2009).

To effectively study the half-reactions of a flavoenzyme, a completely anaerobic system is required to prevent oxygen interference. Anaerobic chambers establish and maintain an anaerobic workspace that can be used to perform enzyme assays to study flavoenzyme half-reactions. This chapter discusses the application of an anaerobic chamber to control the concentration of oxygen when performing these assays with a focus on using stopped-flow spectrophotometry.

2. ANAEROBIC CHAMBERS

Anaerobic chambers maintain a hypoxic atmosphere inside of a hermetic container permitting strictly anaerobic experiments to be performed without the risk of oxygen contamination. These chambers are regularly used in microbiology to grow anaerobes and in anaerobic biochemistry to study oxygen sensitive biomolecules (Crack, Green, Thomson, & Le Brun, 2014; Lange & Ahring, 2001). In protein chemistry, their use has been applied for preparation of reagents, purification, storage, crystallization, and to perform enzyme assays (Echavarri-Erasun, Arragain, & Rubio, 2014; Lee, Kwon, Kim, & Rhee, 1998; Tsai & Tainer, 2018). With flavoenzymology in particular, these chambers allow an investigator to work in an anaerobic environment ideal for studying flavin reduction and oxidation.

2.1. Anaerobic chamber components

All anaerobic chambers share similar elements for their operation. Because these chambers are much larger than other options (e.g., anaerobic jars and glovebags), they require a more permanent set up including the use of an antechamber, catalytic filters, an oxygen and hydrogen monitor, and oxygen free gases. The most common features of an anaerobic chamber are described in Fig. 2 and in the following bullets.



Fig. 2. Top panel: A diagram of an anaerobic chamber displaying the key components. The figure is modeled from a COY Laboratories Type A vinyl anaerobic chamber. Bottom panel: an image of a COY Laboratories Type A vinyl anaerobic chamber. Chamber dimensions are $36'' \times 76''$ for the full chamber with a $32'' \times 59''$ workspace.

- Antechamber: A chamber situated to the side of the anaerobic chamber with one airlock door inside of the anaerobic chamber and one accessible from the outside. The antechamber is essential for transferring solutions without causing oxygen contamination. Every time material is transferred into or out of the chamber, the antechamber undergoes a cycle of vacuum and gas purges to bring the antechamber to anaerobic conditions.
- *Glove ports*: Ports built into the chamber shell that allows a user to access material inside the anaerobic chamber. Glove ports can vary depending on the chamber model with a significant difference being

between gloved ports compared to gloveless ports. Gloved ports secure interchangeable gloves of varying sizes to the ends of each sleeve. Gloveless ports use a sleeve-vacuum system allowing an investigator to directly interact with materials inside the chamber. Depending on the size of the chamber, multiple ports can be added.

- *Vacuum pump*: A mechanical pump required to remove air from the antechamber when establishing anaerobiosis. The pump is also be used to remove gases from the anaerobic chamber to regulate pressure or to remove oxygen contamination. In the example model shown in Fig. 2, the vacuum pump brings the antechamber to a vacuum pressure of 20 in Hg.
- Oxygen free gas tanks: Oxygen free gas is used to fill the chamber and antechamber. The atmospheric conditions used in most chambers consist of a 5% hydrogen and 95% nitrogen mix that can be created in the laboratory with a gas mixer, or purchased premixed.
- *O*₂/*H*₂ *monitor*: A device that shares dual purpose in detecting oxygen at ppm and hydrogen as a percentage using different electrochemical sensors.
- *Contaminant filters*: It is required for a chamber to be able to remove oxygen, water, and hydrogen sulfide from the chamber atmosphere. Commonly, a regenerative catalyst is used to convert the contaminants into inert products. Section 2.2 discusses this process in greater detail.
- Other equipment: Inside each anaerobic chamber, some basic equipment is often including to improve experimentation. A shelving unit provides extra storage space for chemicals and materials, a power strip is needed so equipment can be used, and a stir plate is commonly added to mix solutions.

Most anaerobic chambers follow a similar process to establish and maintain anaerobiosis. A vacuum pump and oxygen free gasses are used to create a closed circulating system that removes air from the chamber and fills it with a 95% nitrogen/5% hydrogen mix to establish an anaerobic atmosphere. Contaminant filters are then used to maintain these conditions while a monitor detects the levels of oxygen and hydrogen in the chamber. Because the hydrogen concentration decreases due to the reaction in the filters (see Section 2.2.1), the level of hydrogen needs to be maintained between 1.5% and 4% by cycling the chamber with vacuum and mixed gas. Each anaerobic chamber manufacturer provides detailed

instructions that should be followed when setting up this equipment. In this chapter, a COY vinyl chamber is used in the methods described.

2.2. Removing contaminants from anaerobic chambers

Anaerobic chambers operate as a closed system leading to a buildup of unwanted side products that can ruin assays or damage electronics if not properly maintained. For this reason, a filtration system is required to continuously remove contaminants including oxygen, water, and hydrogen sulfide. This section discusses some of the equipment and techniques used to remove contaminants from an anaerobic chamber.

2.2.1. Maintaining chamber anaerobiosis

Oxygen contamination is a serious concern as even low levels can interfere with anaerobic stopped-flow experiments. This problem is addressed using a catalyst that reacts with oxygen forming nonreactive byproducts. COY Laboratories utilize palladium chloride coated alumina beads to react with hydrogen and oxygen to form water (Eq. 1). These beads are placed inside of a metal cassette called a Stak-Pak and inserted into a box that uses a fan to circulates gasses through the Stak-Pak filter. Other metals can also be used to react with oxygen. For example, Belle Technologies use copper/magnesium silicide pellets to react with oxygen forming copper oxide (Eq. 2). This specific reaction does not require hydrogen or form water as a byproduct. The use of copper as a reagent does mean that the copper catalyst is more quickly oxidized than the palladium catalyst used by COY Laboratories.

$$2H_2 + O_2 \xrightarrow{Pd} 2H_2O \tag{1}$$

$$O_2 + 2Cu \to 2CuO \tag{2}$$

Both reagents described can be regenerated back to their reduced forms using a chemical oven heated to 125–200 °C for 2 h. This allows the catalysts to be reused providing a cheap method for oxygen removal. Both examples maintain atmospheric oxygen concentrations below 2 ppm.

2.2.2. Maintaining humidity and removing hydrogen sulfide

Water vapor is produced from the evaporation of liquids stored in the chamber, exterior moisture entering the chamber, and from oxygen removal depending on the catalyst used. Without any form of regulation, the humidity inside of an anaerobic chamber will reach saturating vapor concentrations causing water condensation that will deactivate metal catalysts used to remove oxygen and potentially damage electronics component of instruments housed in the anaerobic chamber (Selmer, 2005). Different chemicals can been used to maintain humidity including sodium hydroxide, calcium hydroxide, and aluminum oxide. One of the more popular desiccants used is alumina as it readily absorbs water from circulating air and is easily regenerated using a chemical oven. Beside chemical catalysts, a dehumidifier can also be used.

Hydrogen sulfide (H_2S) is an anaerobic digestion byproduct of sulfate reducing organisms. This chemical is highly explosive and is corrosive, damaging metals, electronic sensors, and the chamber surface with prolonged exposure. If working with anaerobic organisms, it is essential to have an activated carbon and/or iron oxide column present to remove H_2S from the chamber atmosphere.

2.3. Flexible chambers compared to rigid chambers

Anaerobic chambers can be described as either flexible or rigid. Flexible chambers are made of polyvinyl material attached to an aluminum frame, while rigid chambers can be made of clear acrylic or polycarbonate material. Both types of chambers are regularly used for a variety of applications, and cover many of the same requirements including use of an antechamber, catalysts, and oxygen free gases. When purchasing a new chamber, however, there are significant differences that should be considered between these chamber types (Table 1).

Flexible chambers	Rigid chambers
Flexible chamber minimizes the amount of gaswasted on regulating pressure Chamber is clear on all sides Working area is generally larger Optimal ergonomics Cheaper to maintain	Can store oxygen sensitive items for long periods of time Durable structure Modular units allow specialization Limited user movement Wastes more gas due to pressure regulation

TABLE 1 A summary of flexible anaerobic chambers compared to rigid anaerobic chambers.

3. INTRODUCTION TO STOPPED-FLOW SPECTROPHOTOMETRY

Stopped-flow spectrophotometry is used to monitor fast enzymatic reactions as it provides rapid mixing of solutions and records spectra on a millisecond time scale. In flavoenzymology, this instrument is especially useful to determine the rate constants associated with flavin reduction and oxidation. The use of stopped-flow spectrophotometry to study the mechanism of flavoenzymes has contributed to elucidating many flavoenzyme mechanisms and to the detection of intermediates in the catalytic cycle (van Berkel, Benen, Eppink, & Fraaije, 1999).

3.1. Components of a stopped-flow spectrophotometer

Most stopped-flow spectrophotometers contain similar components that rapidly mix solutions and record multiple spectra using different types of detectors. Each stopped-flow spectrophotometer provides a sample handling unit used to rapidly mix samples, a lamp with a power source, and a control units, and a computer. When discussing stopped-flow spectrophotometry, this chapter will be referring to the SX20 series from Applied Photophysics. Specifications of this instrument are provided along with a general description of the instrument parts in the following bullets and in Fig. 3.

- *Electronics communication unit*: This unit contains electronics that control the various components of the stopped-flow apparatus and processes the information from the detectors connected to the sample handling unit.
- *Lamp power source*: Provides the energy needed to power the stopped-flow spectrophotometer lamp.
- Lamp housing: A xenon lamp is commonly used.
- *Monochromator*: This unit is used to select specific wavelengths using a diffraction grating. For Applied Photophysics, a 250 nm holographic diffraction grating is used, which provides a wavelength range of 190–850 nm. The unit is also equipped with adjustable entrance and exit slit widths (0.25–2 mm).
- Sample handling unit (SHU): The SHU performs rapid mixing with one or two drives mixing up to four different solutions. Pneumatic



Fig. 3. Diagram of a stopped-flow spectrophotometer modeled from the SX20 unit from Applied Photophysics. The sample handling unit can be configured into a single-mixing mode or a double-mixing mode utilizing the four syringe ports (A–D) and two drives (1 and 2). For the single-mixing mode, only drive 1 is used to mix the solutions from syringe ports (A) and (B). For the double-mixing mode, both drives are used with drive 2 mixing the solutions from syringe ports (C) and (D) in an aging loop before drive 1 mixes the solutions from (A) and (B) with the solution in the aging loop. Detectors with photomultiplier tubes would be attached to the back of the sample handling unit.

force is used to push the drives and mix solutions. A stop syringe controls the exact volume pushed during each drive by expelling an adjustable volume from its syringe providing only enough room to accommodate the desired volume. The SHU is equipped with a quartz optical cell with a pathlength of 2 or 10 mm.

• *Detector*: The SHU can be equipped with interchangeable fluorescence or absorbance photomultiplier, or a photodiode array (PDA) detector.

3.2. Anaerobic stopped-flow spectrophotometry

When used under anaerobic conditions, a stopped-flow spectrophotometer can study changes to the flavin spectra that are associated with reductive and oxidative half-reactions. Anaerobic chambers serve as an effective way to perform anaerobic stopped-flow spectrophotometry assays as they provide the ability to maintain a stable anaerobic environment for preparing and performing enzyme assays. These chambers also reduce the amount of time and gas spent deoxygenating solutions as it only requires an anaerobic stock solution which can be stored inside of the chamber for days at a time. The anaerobic environment allows a more relaxed preparation and performance of enzyme assays. This setup can also perform anaerobic assays without relying on an oxygen scavenger (glucose oxidase) or a chemical reducer (sodium dithionite) to remove residual oxygen. Using chambers for stopped-flow spectrophotometry is a useful method that is well-established in the flavoenzyme community (Knight & Scrutton, 2002; Robinson, Badieyan, & Sobrado, 2013; Sucharitakul, Wongnate, & Chaiyen, 2011; Yu, Hausinger, Tang, & Xu, 2014). Even with its benefits, anaerobic chambers also take up significant space in a lab and their initial cost and maintenance are expenses that need to be considered when comparing the two methods (Table 2).

> 4. SETTING UP, PREPARING, AND PERFORMING STOPPED-FLOW SPECTROPHOTOMETRY ASSAYS INSIDE OF AN ANAEROBIC CHAMBER

Performing anaerobic assays with a stopped-flow spectrophotometer inside of an anaerobic chamber requires careful set up of the instrument to allow easy handling once inside the chamber. It is also important to establish a method for preparing deoxygenated solutions that consistently produce anaerobic buffers with limited risk of contamination. This section describes the process of setting up a stopped-flow spectrophotometer inside of an anaerobic chamber, setting up Schlenk line appara-

TABLE 2	Summary	of the benefit	s and d	lisadvantage	s of u	using an	anaerobic	chamber	for
stopped-flo	ow spectroj	photometry.							

Benefits of using an anaerobic chamber

Stable anaerobic environment

Easy preparation of anaerobic solutions from stock solutions

Lower risk of O2 contamination, leading to less experimental error

Less time and materials spent making the solution anaerobic

Instrument accessibility is not limited by being inside of the chamber

Disadvantages of an anaerobic chamber

Costs of purchase and maintenance

Requires a large footprint in the lab

The stopped-flow equipment takes up space in the chamber that could be used for other things

Limited mobility

Continuous chamber maintenance required

tus, deoxygenating buffers, validating solution anaerobiosis, preparing samples for experimentation, and measuring the reductive and oxidative half-reactions of a flavoenzyme using stopped-flow spectrophotometer. While the described methods have proven to be successful in our lab, considerations should always be made to optimize conditions specific to each investigator.

4.1. Setting up a stopped-flow spectrophotometer inside of an anaerobic chamber

When setting up a stopped-flow spectrophotometer inside of an anaerobic chamber, it is important to consider the placement of each component in order to optimize operation and handling. This section provides recommendations for arranging an SX20 Applied Photophysics stopped-flow spectrophotometer inside of a COY Laboratories Type A vinyl chamber. Both the chamber and the stopped-flow model are two of the more popular designs in their respective categories. For this reason, they serve as a good example of how a stopped-flow spectrophotometer can be operated inside of a chamber. It should also be mentioned that hard shell anaerobic chambers are compatible for stopped-flow spectrophotometry studies as well (Knight & Scrutton, 2002).

4.1.1. Materials

- Stopped-flow spectrophotometer (Applied Photophysics).
 - Sample handling unit (SHU).
 - Electronics communication unit.
 - Lamp power source.
 - Lamp holder.
 - Monochromator.
 - Detectors: Photodiode array, absorbance, or fluorescence.
 - Computer.
- Anaerobic chamber (COY Laboratories)
- A large sturdy table or other workspace.
- Water temperature regulator (Fisher Scientific).
- Nitrogen/hydrogen (95%/5%) gas mix.
- Gas regulator.
- Rubber sealant.
- Electrical tape.

4.1.2. Procedure

- 1. Situate the anaerobic chamber next to a table or bench that is capable of holding stopped-flow spectrophotometer equipment.
- 2. Remove the chamber cap and unseal all wire ports.
- **3.** Place a sturdy Teflon plastic sheet underneath a disconnected SHU. This is to protect the chamber from damage. Carefully, place the instrument in the chamber, and angle the SHU in a manner so that it is easy to reach from the glove ports.
- 4. If not already in the chamber, place the catalyst boxes, a shelving unit, and a standard power strip inside of the chamber. Place all detectors that will be used with the SHU into the chamber as well.
- 5. Place the other stopped-flow equipment and the water temperature regulator in the space next to the chamber. Fig. 4 shows an example of how this equipment can be placed.
- 6. Secure a mixed gas tank to a wall. Attach the gas regulator to the tank and connect it to the SHU using gas tubing. Make sure to thread the connection through a wire port.
- 7. Carefully begin to make connections starting with the wires going through the wire ports. A suggested setup is shown in Fig. 4.
- **8.** Finish setting up the other connections of the instrument. Make sure a power strip for the equipment is accessible.
- **9.** Test the operation of the equipment by turning it on and performing a dry run. If any component is difficult to reach or awkward to handle, adjust the equipment accordingly.
- **10.** Once the equipment has been optimized, seal the ports with rubber sealant. Let the sealant dry for 24 h.
- **11.** Return the chamber cap to its proper place and seal the entire rim multiple times with electrical tape.
- 12. Vacuum air out of the chamber as much as possible and refill it with 95%/5% nitrogen/hydrogen mixed gas. Repeat until the O_2/H_2 monitor records a percent hydrogen between 1.5% and 4% and 0 ppm O_2 .
- 13. For a few hours, watch the percentage of hydrogen to see if it is rapidly decreasing. This would suggest leak in the system. Remove sealant and chamber cap and repeat steps 9–12 if a leak is suspected.



Fig. 4. A stopped-flow spectrophotometer inside an anaerobic chamber. The top panel represents a diagram of the described setup from a top down perspective with the specific wires in each port described. Lower left panel: materials inside of the anaerobic chamber including an Applied Photophysics SHU, a magnetic stir plate, a wireless computer mouse, COY Laboratories catalyst boxes, and an O_2/H_2 monitor. Lower right panel: the exterior of the chamber with the stopped-flow spectrophotometer setup including electronics unit, lamp power supply, lamp holder, monochromator, computer, water bath temperature regulator, anaerobic chamber, and gas tanks.

14. Place equipment needed to perform assays with a stopped-flow spectrophotometer inside of the chamber. This includes a stir plate, Allen wrenches to remove detectors, a wireless mouse (Fig. 4), sample syringes, sample filters, 2 M nitric acid, and deionized water.

4.1.3. Notes

- 1. Make sure there are no oxygen sensitive materials inside the anaerobic chamber when opening the chamber to the room atmosphere.
- 2. If one detector will be used frequently (e.g., absorbance), it is recommended to attach the detector to the SHU before moving the instrument into the chamber.
- **3.** When placing equipment, keep in mind the distance between connections. For example, the reach of the fiber optic light guide is about 1 m, so the orientation of the monochromator and the SHU should be within this range so that an extension is not required.
- **4.** In the setup shown in Fig. 4, there are three wire ports close to the SHU providing access from multiple sides.
- 5. Be creative with what is tested in the dry run in step 8. Try changing detectors or switching the pathlength while using the glove ports, change the monochromator entrance and exit slit widths, and turn on the water regulator to check for leaks. Operation of the stopped-flow spectrophotometer equipment should not be hindered by being in the chamber. A person should still be able to adjust the settings with little difficulty. The arrangement should also allow plenty of space to put supplies and perform assays without crowding.
- 6. Make sure there is plenty of slack with the wires and lines inside of the chamber to allow easy removal.
- 7. Include a wireless mouse inside of the chamber for easier operation of the computer during experimentation.
- 8. With a careful setup, the SHU can be removed from the chamber for fixing or cleaning without breaking the rubber seal on the wire ports. This saves time and materials as this seal takes up to a day to be fully hardened.

4.2. Setting up a Schlenk line on a cart

An essential part of preparing assays under anaerobic conditions is the process of removing oxygen from buffers, substrate solutions, and enzyme stocks. There are several methods used to deoxygenate buffers, including sonication, sparging, bubbling, boiling with an inert gas head-space, or using a vacuum gas manifold (a Schlenk line) (Butler, Schoonen, & Rickard, 1994; Lowe, Fisher, & Thorneley, 1993; Romero, Robinson, & Sobrado, 2012; Selmer, 2005). This chapter specifically describes using a Schlenk line to obtain anaerobic solutions with this section focusing using a Schlenk line set up on a cart. This method allows the system to be used in various locations without disassembling or dismounting.

A Schlenk line consists of two glass manifolds specially made to withstand changes in pressure and temperature with multiple valves fitted with double-oblique stopcocks. To use a Schlenk line, one manifold is kept under vacuum while the other is filled with a pure inert gas, typically argon. Airtight flasks are attached to the Schlenk line by a tube, and dissolved oxygen is removed through cycles of high vacuum and inert gas purges. These two conditions in combination with rapid stirring exchanges the gasses from a solution with oxygen free inert gas making it anaerobic. A suggested set up of a Schlenk line for degassing buffers is described along with a visual example (Fig. 5).

4.2.1. Equipment

- Large cart.
- Aluminum support frame.
- Glass dual manifold (Schlenk line).
- Norprene tubing.
- Vacuum hosing.
- Stainless steel hose clamps (MSC Industrial Supply Co) and a screwdriver.
- Glass oil bubbler.
- Glass high vacuum stopcock.
- Vacuum and gas pressure gauges.
- Vacuum pump (Welch).
- Dry ice cold trap (Labconco).



Fig. 5. Setup of a Schlenk line discussed in Section 4.2.2. (A) The overall setup. The bottom of the cart contains a vacuum pump and cold trap. The top of the cart contains two stir plates and an aluminum structure holding a Schlenk line with the attached bubbler and gauges. A high purity argon gas tank connected to an oxygen trap (Agilent) is in the background. (B) The Schlenk line is secured to the aluminum structure using zip-ties. Norprene tubing is attached to the end of the stopcock valve with hose clamps, and each tube is marked with a different colored tape. (C) The bottom of the cart with the vacuum pump (Welch) and cold trap (Labconco). Vacuum hosing attaches the cold trap to the inlet of the vacuum pump. Hosing to the Schlenk is shown in the back going through a hole in the cart top. (D) Close up image of the oil-bubbler and high-vacuum stopcock attached to the Schlenk line. Each attachment is secured with hose clamps. (E) Close up image of the vacuum and gas gauges secured to the Schlenk line with hose clamps.

- An oxygen trap (Agilent).
- Magnetic stir plate.
- Flexible copper tubing.
- Plastic zip ties.
- Vacuum grease.

4.2.2. Procedure

- 1. Assemble an aluminum frame to hold the Schlenk line. Drill the ends of the frame into the cart to secure it.
- 2. Secure the Schlenk line to the top of the frame using zip ties (Fig. 5B).
- **3.** Lubricate the stopcocks with vacuum grease and firmly tightened to the manifold using an O-ring and plastic nut.

- 4. Secure Norprene tubing (~ 3 ft.) to the stopcocks and secure with hose clamps (Fig. 5B).
- 5. To the exits of the Schlenk line, secure a gas gauge and a vacuum gauge to their respective manifolds using Norprene tubing with metal hose clamps (Fig. 5E).
- 6. Place the vacuum pump on the bottom shelf of the cart.
- 7. From the intake of the vacuum pump, secure vacuum hosing to a cold trap.
- **8.** Attach the cold trap to the inlet of the vacuum manifold using a combination of vacuum hosing and Norprene tubing (Fig. 5C). Secure the tubing with hose clamps.
- **9.** To the entrance of the argon gas manifold attach a glass oil bubbler using Norprene tubing and secure it with metal hose clamps (Fig. 5D).
- **10.** Secure a high purity argon gas tank to a wall and attach a gas regulator.
- **11.** Use the flexible copper tubing to attach the gas tank to an oxygen trap.
- **12.** Attach copper tubing from the trap to rubber tubing with a hose clamp. Connect the tubing to a high vacuum stopcock (Fig. 5D).
- **13.** From the stopcock, connect the rubber tubing to an oil bubbler and secure with a clamp.
- 14. On the top of the cart, place two stir plates with clamps attached to the aluminum frame.

4.2.3. Notes

- 1. All oxygen contaminants in the inert gas should be removed by passing the gas through an oxygen trap. Oxygen traps remove oxygen and sulfur from non-oxidizing gases in bulk by using a reactive metal catalyst. In Fig. 5, the oxygen trap can be seen secured to the wall. The gas should also pass through an oil-bubbler containing water for (i) gas hydration and (ii) detection of gas flow.
- 2. Make sure the vacuum pump has the proper amount of oil. Remember to change the oil every couple months or more often depending on use.

4.3. Preparing anaerobic buffers

This method describes the removal of oxygen from a buffer using a Schlenk line with high purity argon gas. This is useful for kinetic analysis on a stopped-flow spectrophotometer and can be used to prepare anaerobic buffers for other assays as well.

4.3.1. Equipment

• Schlenk line setup (Section 4.2.2).

4.3.2. Buffers and reagents

- Buffer solution, 100 mL.
- High purity argon gas.
- Dry ice for the cold trap.

4.3.3. Procedure

- 1. Measure 100 mL of a buffer and place into a 250 mL Büchner flask with a stir bar.
- 2. Cover the flask with a (i) rubber septum or (ii) rubber stopper.
- **3.** Attach the Schlenk line to the side arm of the Büchner flask. If using the designed tubing described in Fig. 6, make sure there is a tight seal by taping the tube to the side of the flask.
- 4. Place the flask onto a stir plate and begin stirring.
- 5. Put dry ice into the cold trap and start the vacuum pump.
- 6. Turn on the argon gas and adjust the pressure to 5 psi.
- 7. Place the solution under vacuum for 4 min.
- 8. Purge with argon for 1 min.



Fig. 6. Left panel: disassembled set up of connectors used to detach and transfer anaerobic solutions to the anaerobic chamber without oxygen contamination. (A) Norprene tubing, (B) straight thru valve, (C) shutoff valve, (D) nylon hose clamps. Right panel: assembled setup attached to a Büchner flask.

- 9. Repeat for four cycles for a total of 20 min.
- **10.** Turn off vacuum after the last argon purge. Leave the flask under positive pressure from the argon.
- **11.** Detach from the Schlenk line, and transfer flask into the anaerobic chamber.

4.3.4. Notes

- 1. Be sure to seal the stopper/septum with electrical tape. The rubber stopper risks leaking oxygen into the flask during argon purges, and the septum will pop off in the antechamber when under negative pressure.
- 2. The buffer loses 4% volume when using this method.
- **3.** The vacuum pressure should be approximately 28 in Hg. Anything higher might not provide enough vacuum to remove dissolve oxygen.
- 4. Make sure to always have dry ice in the cold trap, and remove all condensed solvent after use. If this is not done, water will collect inside of the vacuum oil and corrode the interior of the pump.

4.4. Transferring anaerobic solutions

Methods to transfer anaerobically prepared solutions to the anaerobic chamber should be considered as even minute exposure to air can cause oxygen contamination. To address this concern, Norprene tubing can be retrofitted to detach from the Schlenk line and move with the flask into the chamber (Fig. 6). This simple design minimizes the risk during transfer from the Schlenk line into the anaerobic chamber.

4.4.1. Equipment

- Norprene tubing.
- In-line hose barb LC series chrome plated brass inline body shutoff valve (Colder Products).
- In-line hose barge LC series plates brass insert, straight thru valve (Colder Products).
- Nylon hose clamps.

4.4.2. Procedure

1. Cut a short segment of tubing that fits tightly around the arm of a Büchner flask (~ 6 in.).

- 2. Insert a shut off valve on the end of the tubing segment.
- 3. Secure the valve with a nylon hose clamp until it is airtight.
- 4. On the end of a long tubing attached to a Schlenk line, insert the straight thru valve and secure it with a hose clamp.

4.4.3. Notes

1. This system uses a shutoff valve to seal the end of the tube preventing oxygen from entering the flask from the sidearm of the Büchner flask during transfer.

4.5. Addition of enzymes to maintain anaerobic solutions

After a buffer has been made anaerobic, addition of enzymatic oxygen scavengers is sometimes used to maintain anaerobiosis. While reducing agents including sodium dithionite and titanium(III) citrate can be used to remove oxygen, these chemicals will also reduce flavoproteins interfering with the kinetic studies. Oxygen consuming enzymes do not react with the flavin cofactor allowing them to be used in assays with minimal interference. This section describes two enzyme systems that are regularly used to maintain anaerobic solutions.

Glucose oxidase (GO) is a flavoenzyme that converts glucose in the presence of oxygen to gluconolactone and hydrogen peroxide. This enzyme is easy to isolate from Aspergillus niger and Penicillum species making it commercially available as a lyophilized powder. The stability of this enzyme along with its common substrate has caused GO to be used for a broad range of applications including glucose detection, food preservation, and biotech (Bankar, Bule, Singhal, & Ananthanarayan, 2009). Among these uses, GO is regularly used as an oxygen scavenger to remove residual oxygen and maintain anaerobiosis. GO is active in most aqueous buffers with a pH range of 3.4-7.5 and is optimal at a temperature between 40 and 45 °C (Selmer, 2005). This enzyme is also used to scrub stopped-flow spectrophotometers of oxygen before experimentation. GO it is not always the ideal choice for oxygen scavenging as it produces hydrogen peroxide. GO also has a high K_m that demands a high concentration of glucose to be added to the activity buffers (Swoboda & Massey, 1965).

Another enzyme system that can be used to maintain anaerobic solutions is protocatechuate dioxygenase (PCD). PCD converts protocatechuate to β -carboxy-*cis*, *cis*-muconic consuming a molecule of oxygen without the creation of hydrogen peroxide or any other reactive species. PCD has been shown to be active at a pH 6–9 and a temperature range of 15-25 °C making it very useful for assays performed at room temperature and for scrubbing instruments (Patil & Ballou, 2000). Patil and Ballou in 2000 showed stable reduction of free flavin with sodium dithionite in a solution of 6.5 mg/mL PCD and 0.4 mM protocatechuate. The PCD system also has been shown to produce solutions with lower dissolved oxygen concentrations than GO (Aitken, Marshall, & Puglisi, 2008). While this enzyme is an excellent substitute, it is the more expensive alternative, with 25 units of PCD costing \$298 from Sigma Aldrich compared to 10 kU of GO for \$45.

Our lab regularly uses GO for the preparation of enzyme assays with a stopped-flow spectrophotometer. Preparation of this enzyme is a simple procedure that can be adjusted to fit specific lab requirements. The following sections describe the technique for preparing a GO solution for scrubbing a stopped-flow spectrophotometer. For a description of adding GO to assay buffers, refer to note 1 (Section 4.5.4).

4.5.1. Equipment

- Schlenk line setup (Section 4.2.2).
- Stopped-flow spectrophotometer setup (Section 4.1.2).
- 50 mL conical tube.
- Four disposable 5 mL luer lock syringes.

4.5.2. Buffers or reagents

- GO from Aspergillus niger (> 100,000 U/g, Sigma-Aldrich).
- GO buffer: 100 mM sodium acetate, pH 5.0 (Fisher Scientific).
- Glucose (Fisher Scientific).

4.5.3. Procedure

- 1. Measure 100 mL of 100 mM Na-acetate, pH 5.0 buffer and pour into a Büchner flask.
- **2.** Deoxygenate buffer using the procedure described in Section 4.3.3.
- **3.** Measure 5 mg of GO and 900 mg of glucose and place components inside of a 50 mL conical tube.
- 4. Transfer tube into the anaerobic chamber with buffer.

- 5. Dissolve enzyme and glucose in 50 mL of anaerobic 100 mM Na-acetate, pH 5.0 buffer. Final concentration will be $0.6 \mu M$ GO and 100 mM glucose.
- 6. Draw anaerobic buffer into four luer lock syringes.
- 7. Replace the sample syringes on the SHU with the syringes filled with GO dissolved in 100 mM Na-acetate, pH 5.0 buffer.
- **8.** Flush the system with 100 mM Na-acetate, pH 5.0 buffer with GO by either using the drive option on the program or manually.
- **9.** Leave the SHU in 100 mM Na-acetate, pH 5.0 buffer with GO overnight.

4.5.4. Notes

- 1. GO can also be mixed with anaerobic buffers to maintain anaerobiosis during enzyme assays. For this application, 3 mg of GO and 450 mg of glucose should be dissolved in 50 mL of assay buffer making a final concentration of $0.4 \,\mu\text{M}$ GO and 50 mM glucose.
- 2. For more information, this technique is described in another publication from our lab (Romero et al., 2012).

4.6. Determining oxygen concentration in deoxygenated buffers to validate anaerobiosis

When preparing anaerobic buffers, it is important to quantitate the concentration of oxygen present in a solution to determine how effective the deoxygenating process being used is. This technique is very helpful especially when troubleshooting for oxygen contamination. Here, the use of the indicator methyl viologen will be discussed. Methyl viologen is a redox-active heterocyclic compound that forms a stable structure with a strong absorbance when reduced with sodium dithionite. This agent is very reactive with oxygen and changes from clear to dark blue between its oxidized and reduced forms (Selmer, 2005). The data shown in Fig. 7A were collected using the method described here.

4.6.1. Equipment

- Anaerobic chamber (COY Laboratories).
- Schlenk line setup (Section 4.2.2).
- Anaerobic cuvette (Section 5.3).
- UV-visible spectrophotometer (Agilent).



Fig. 7. Oxygen concentration of 100 mM NaPO₄, pH 7.5 after cycles of argon/vacuum as a function of time.

4.6.2. Buffers and reagents

- Aqueous buffer, 100 mL.
- Methyl viologen (Sigma-Aldrich). Dissolve 26 mg into 10 mL anaerobic deionized (DI) water for a stock solution of 10 mM.
- Potassium hexocyanoferrate (Sigma-Aldrich). Dissolve 33 mg into 10 mL anaerobic DI water for a stock solution of 10 mM.
- Sodium dithionite (Merck). Dissolve 17.4 mg into 10 mL of anaerobic DI water for a stock solution of 10 mM.
- Oxygen free argon, nitrogen, and nitrogen/hydrogen (95%/5%) gasses.

4.6.3. Procedure

- 1. Prepare deoxygenated buffer following the protocol in Section 4.3.3.
- 2. Transfer solutions into the anaerobic chamber.
- 3. Determine the concentration of sodium dithionite by measuring the reduction of potassium ferrocyanide. Use an anaerobic cuvette to measure the concentration of 1 mM potassium ferricyanide on a UV-vis spectrophotometer. Mix 1 mM potassium ferricyanide with 50 μ M of the sodium dithionite stock. Transfer the solution to an

anaerobic cuvette and record the absorbance with a UV-vis spectrophotometer.

- 4. From the change in absorbance determined from step 2, calculate the amount of potassium ferricyanide consumed using the Beer-Lambert equation ($\varepsilon_{405} = 1.04 \text{ mM}^{-1} \text{ cm}^{-1}$). From this, calculate the concentration of sodium dithionite.
- 5. Prepare a 2.5 mM methyl viologen reduced stock by mixing it with 2.5 mM sodium dithionite and anaerobic buffer.
- 6. Mix 50 μL of the reduced methyl viologen stock with 1 mL of anaerobic buffer stock and 2 mM sodium dithionite. This solution serves as an anaerobic control. Transfer the solution to an anaerobic cuvette and record the absorbance with an UV-vis spectrophotometer.
- 7. Mix 50 μ L of the reduced methyl viologen stock with 1 mL of anaerobic buffer with no sodium dithionite added. This solution serves as the sample to be compared to the anaerobic control. Transfer the solution to an anaerobic cuvette and record the absorbance with a UV-vis spectrophotometer.
- 8. Determine the change in absorbance between the anaerobic control and the sample from steps 6 and 7. From this change, calculate the amount of methyl viologen oxidized ($\varepsilon_{605} = 13.7 \text{ mM}^{-1} \text{ cm}^{-1}$) (Watanabe & Honda, 1982). Calculate the concentration of residual oxygen in the buffer by determining how much methyl viologen was oxidized.

4.6.4. Notes

- 1. Sodium dithionite will decompose, so it is important to prepare a fresh solution and measure the exact concentration with reducing potassium ferricyanide each time.
- 2. There are different colorimetric indicators that can be used for testing anaerobic solutions. These chemicals vary depending on stability, extinction coefficients, and the color changes between their reduced and oxidized forms. For a detailed explanation of the types of electron acceptors that can be used to determine oxygen concentrations, refer to table 5.2 from Selmer (2005).
- **3.** If the oxygen contamination is high, the methyl viologen will completely oxidize turning the solution clear. To get an exact measure-

ment of oxygen concentration, the methyl viologen concentration can be increased to a point where it is no longer fully oxidized.

4. This method can also quantitate the oxygen concentrations of oxygenated buffers used to study flavin oxidation. To get an exact measurement of the oxygen concentration in this situation, it is recommended to dilute the oxygenated buffer with anaerobic buffer to a point where it does not oxidize all of the reduced methyl viologen.

The method described has been used to determine anaerobiosis of buffers prepared using the method from Section 4.3.3. Reduced methyl viologen stock was mixed with aqueous buffer (100 mM NaPO₄, pH 7.5) deoxygenated at different time points from 0 to 60 min. The results from this experiment show that most of the oxygen was removed from the buffer after 10 min and was completely anaerobic after 20 min (Fig. 7).

4.7. Preparing anaerobic enzyme solutions

The day of experimentation, several samples have to be carefully prepared before analysis can begin. Even with correct buffer preparation and scrubbing of the instrument, using solutions that have not been appropriately deoxygenated will ruin the enzyme assays. As many substrate and cofactors are sold from retailors as solids, these can be dissolved in anaerobic buffer removing any concern of oxygen contamination. However, this typically cannot be accomplished with enzymes as only a few are stable when lyophilized. While degassing methods can be applied, the rigorous method described in Section 4.3.3 would lead to protein denaturation. To prevent this, the Schlenk line can be used in a gentle manner to deoxygenated enzyme solutions. Since the volume used with protein stocks is much smaller than with buffers, the enzyme solutions can be deoxygenated within a few minutes.

4.7.1. Equipment

- Schlenk line setup (Section 4.1.2).
- A plastic male luer lock with hose barb.
- 5 or 20 mL serum flask with a rubber plug and peel off metal cap (Wheaton).
- Small plastic container (e.g., an empty tip box).
- A small stir bar.
- Disposable needle (BD).

4.7.2. Buffers and reagents

- Ice.
- Dry ice.
- Enzyme stock solution.
- High purity argon gas.

4.7.3. Procedure

- 1. Thaw the required amounts of enzyme stock solution on ice.
- 2. Place enzyme into a 5 or 20 mL Wheaton flask depending on volume. Add a small stir bar and cap the flask using a crimper.
- **3.** Fill a small plastic container with ice and water. Place the container onto a stir plate below the Schlenk line with the enzyme solution resting in the ice water slurry.
- 4. Secure the flask with a clamp.
- 5. Insert the needle into a male luer-lock secured to the end of the Norprene tubing attached to the Schlenk line. Insert the needle into top of the flask.
- 6. Start stirring the enzyme solution.
- 7. Turn on the vacuum pump and argon gas.
- **8.** Switch the enzyme flask to vacuum for a few seconds until bubbles begin to form. When this is observed, turn the stopcock to argon gas for 15 s.
- 9. Repeat step 8 for 20 cycles.
- **10.** Remove the needle from the flask.
- 11. Place the flask into the antechamber.
- **12.** Dilute enzyme solution with anaerobically prepared buffer to the desired concentration needed.

4.7.4. Notes

- 1. The enzyme solution should be deoxygenated as a concentrated stock and diluted inside of the chamber with anaerobic buffer.
- 2. When inserted into the flask, the needle should occupy the headspace and not submerge in the solution.

4.8. Preparing anaerobic substrate and cofactor solutions

Substrates and cofactors are commonly used when studying flavoproteins, and require anaerobic preparation for kinetic use. In situations where the ligand is already dissolved, the sample can be diluted with aerobic buffer to the desired concentration and deoxygenated following the protocol in Section 4.7.3. Alternatively, solid substrates and cofactors can be dissolved with anaerobic buffers inside of an anaerobic chamber.

4.8.1. Equipment

- Anaerobic Chamber (COY Laboratories).
- UV-visible spectrophotometer (Agilent).
- Anaerobic cuvette
- Microcentrifuge or conical tubes.

4.8.2. Buffers and reagents

- Pre-prepared anaerobic assay buffer (Section 4.3).
- Cofactor or substrate in a solid form.
- DI water.

4.8.3. Procedure

- 1. Measure the amount of the cofactor/substrate required for the planned experiments. Place the material into a tube with the appropriate size for the desired dilution.
- 2. Transfer tube with the compound into the anaerobic chamber.
- 3. Dissolve cofactor/substrate with anaerobic buffer.

4.8.3.1. Quantifying cofactor/substrate concentration

- When preparing a compound without a chromophore (e.g., L-lysine): determine the concentration of the solution from the exact mass of the compound measured and the volume of buffer it was dissolved in.
- When preparing a compound with a chromophore (e.g., NADH or NADPH): Pipette a 2 μ L aliquot of the compound into a 1.5 mL microcentrifuge tube. Remove the tube from the chamber, and dilute the sample with DI water so the expected absorbance is below one.

Measure the diluted sample with a UV–vis spectrophotometer, and calculate for the concentration using the Beer-Lambert equation.

• When preparing an oxygen-sensitive reagents with a chromophore (e.g., methyl viologen): Dilute an aliquot of the sample with anaerobic buffer so the expected absorbance is below one. Transfer the solution into an anaerobic cuvette and remove from the chamber. Measure the diluted sample with a UV-vis spectrophotometer, and calculate for the concentration using the Beer-Lambert equation.

4.9. Preparing oxygenated buffers

In order to study the oxidative half-reactions of some flavoenzymes, it is important to be able to mix concentrations of oxygen into the enzyme assays. This can be accomplished by preparing an oxygen saturated buffer that can be diluted with anaerobic buffer and used to perform the desired assays. Aqueous solutions contain 280 μ M of dissolved oxygen at 20 °C, and fully saturates at ~ 1.2 mM dissolved oxygen at 0 °C (Rice, Baird, & Eaton, 2017).

4.9.1. Equipment

- Pressure regulator.
- Gas lines with a long metal syringe attached.
- A 400 mL beaker.
- A 250 mL volumetric flask with a rubber septum.
- A disposable syringe (BD).
- A stir bar.
- A stir plate.
- A support stand with clamp.

4.9.2. Buffers and reagents

- Oxygen gas.
- Aqueous buffer.
- Ice.

4.9.3. Procedure

- 1. Measure 100 mL of assay buffer and place into a volumetric round bottom flask with a stir bar.
- 2. Cap the flask with a rubber septum.

- **3.** Prepare an ice water solution in a beaker and place on top of a stir bar.
- 4. Secure the flask in the ice bath using a support stand and clamp.
- 5. Insert a clean disposable needle into the septum and begin stirring.
- 6. Insert a long needle attached to an oxygen gas tank into the buffer.
- 7. Turn the tank on and adjust the pressure to 30 psi. Small bubbles should be forming in the solution.
- 8. Allow the oxygen to bubble for 30 min.
- 9. Remove the disposable needle for venting and wait 10 s.
- **10.** Remove the oxygen line.

4.9.4. Notes

1. Do not use a septum that has been punctured multiple times.

4.10.Monitoring flavin oxidation and reduction with stoppedflow spectrophotometry

The flavoenzyme SidA can be used as an example of performing stopped-flow experiments inside of an anaerobic chamber. This enzyme catalyzes the hydroxylation of the amino group on the side chain of L-ornithine forming N⁵-hydroxy-L-ornithine, which is an important step in siderophore biosynthesis in *Aspergillus fumigatus*. The stopped-flow spectrophotometry setup described in this chapter has been used to extensively study this enzyme (Robinson et al., 2013, 2014, 2015). The following procedure outlines performing oxidative and reductive studies inside of an anaerobic chamber using SidA.

4.10.1. Equipment

- Stopped-flow spectrophotometer setup inside of an anaerobic chamber (Section 4.1.2).
- Schlenk line setup (Section 4.2.2).
- Disposable 5 mL luer lock syringes

4.10.2. Buffers and reagents

- GO buffer: 100 mM sodium acetate, pH 5.0 (Fisher Scientific).
- SidA buffer: 100 mM NaPO₄, pH 7.5 (Fisher Scientific).
- L-Ornithine (Sigma-Aldrich).
- SidA enzyme stock.

• Nicotinamide adenine dinucleotide phosphate (NADPH) (Research Products International).

4.10.3. Procedure

4.10.3.1. Day 1

- 1. Prepare at least 100 mL 100 mM NaPO₄, pH 7.5 buffer (SidA) and 100 mM Na-acetate, pH 5.0 (GO) each.
- 2. Switch the SHU to the desired mixing mode. For this example, the reductive half-reaction uses single-mixing that only uses drive 1 (Fig. 3), while the oxidative half-reaction uses double-mixing that uses drive 1 and 2 (Fig. 3).
- **3.** Prepare anaerobic buffers described in Section 4.3.3.
- 4. Transfer anaerobic solutions into the anaerobic chamber.
- 5. Perform oxygen-scrubbing to the SHU as described in Section 4.5.3.
- 6. Leave 100 mM NaPO₄, pH 7.5 buffer stirring inside of the chamber overnight.

4.10.3.2. Day 2

- 7. Turn on the stopped-flow spectrophotometer lamp and water temperature regulator. Let the lamp warm up for 30 min.
- **8.** Thaw the required amount of enzyme stock and deoxygenated it using a Schlenk line (Section 4.7.3).
- 9. Prepare NADPH stock (Section 4.8.3).

4.10.3.3. Reductive half-reaction

- **10.** Prepare the desired concentrations of NADPH for the assay from the stock solution prepared in step 9.
- **11.** Remove the GO solution by flushing the SHU ports A and B with anaerobic 100 mM NaPO₄, pH 7.5 (Fig. 3).
- **12.** Switch the detection mode to PDA. Collect background using anaerobic 100 mM NaPO₄, pH 7.5 buffer.
- 13. Dilute SidA enzyme stock to $30 \ \mu\text{M}$ and draw up into a luer lock syringe.
- 14. Insert syringe into the SHU port A (Fig. 3). Pull a small volume $(\sim 50 \ \mu\text{L})$ into the SHU sample syringe and flush completely out of the SHU by emptying the stop syringe and driving the syringe. Repeat this two times.

- 15. Obtain spectra of the oxidized flavin for 500 s logarithmically.
- **16.** Draw NADPH assay solution into a luer-lock syringe and insert into the SHU port B (Fig. 3). Repeat the process described in step 15.
- 17. Acquire data for 500 s on a logarithmic scale.

Date collected of SidA reduction will show the flavin peak at 450 nm disappear as it is reduced (Fig. 8A).

4.10.3.4. Oxidative half-reaction

- **10.** Remove the GO solution by flushing the SHU ports A, B, C, and D with anaerobic 100 mM NaPO₄ pH 7.5 (Fig. 3).
- **11.** Switch the detection mode to PDA. Collect background using anaerobic 100 mM NaPO₄, pH 7.5 buffer.
- 12. Dilute the SidA enzyme stock to 60 μM with anaerobic 100 mM NaPO₄, pH 7.5 buffer. Insert the sample syringe into the SHU port C (Fig. 3). Pull a small volume (~ 50 μL) into the SHU sample syringe and flush completely out of the SHU by emptying the stop syringe and driving the syringe. Repeat this two times.
- 13. Prepare a solution of NADPH stoichiometric to the concentration of SidA (64–88 μ M). Draw the solution into a luer-lock syringe. Insert syringe into SHU port D (Fig. 3) and repeat the process described in step 12.



Fig. 8. Experimental spectra showing SidA reduction (A) and oxidation (B). Reduced enzyme is shown in blue (dashed), oxidized enzyme is shown in red (solid), and enzyme when it forms the C4a-hydroperoxyflavin intermediate is shown in green (small dashed). The direction of peak changes at 450 nm is represented with the gray arrows.

- 14. Set the delay for the second mixing at 60 s and acquire spectra of the reduced protein for 500 s on a logarithmic scale. During detection, the flavin spectra should be those of a fully reduced enzyme. Repeat two more times to verify that SidA is reduced and remains reduced for the period of 500 s.
- 15. Prepare oxygenated 100 mM NaPO₄, pH 7.5 buffer as described in Section 4.9.3. The concentration of oxygen should be \sim 912 μ M.
- **16.** Dilute oxygenated buffer with anaerobic buffer to get a range of oxygen concentrations. Replace the syringes containing anaerobic buffer in SHU ports A and B (Fig. 3) with syringes containing the desired oxygen concentration. Repeat the process described in step 12.
- 17. Acquire data. For SidA, the spectra will show a peak formation and decay at 360 nm representing the formation of the C4a-hydroper-oxyflavin intermediate.

Date collected of SidA oxidation will show the formation of a C4a-hydroperoxyflavin at 360 nm followed by appearance of the oxidized flavin peak at 450 nm (Fig. 8B).

4.10.3.5. Clean up

- **18.** Flush the system with water to remove the majority of enzyme solution. Wash the SHU with 2 M nitric acid to oxidize the residual enzyme for at a minimum of 2 h and a maximum of overnight.
- **19.** Thoroughly rinse the instrument with water. If the instrument will not be used for a long period of time, leave the system in 20% ethanol.

4.10.4. Notes

- 1. For oxidation studies, it is usually desired that there are stoichiometric amounts of NADPH to SidA as excess NADPH will react with the oxidized flavin interfering with the initial measurements of flavin oxidation.
- 2. The varied reactants (e.g., NADPH and O_2) should always be studied from low to high concentration.
- **3.** During sample preparation, be sure to move carefully inside of the chamber and not to act in haste.
- 4. When changing syringes on the SHU, enough solution should be inside of the instrument to form a positive meniscus on the syringe

port. This should be the same with the syringe containing the sample before placing it onto the port. A very small amount of the sample should be pushed into the handling unit and flushed out before the sample is fully drawn into the instrument. This process helps limit the risk of bubbles being introduced into the instrument which will disrupt the spectra readings.

- 5. A small volume of the sample in the syringes should be pulled into the SHU and flushed three times before conducting a reading to make sure that the sample in the cell is not diluted.
- 6. When transferring samples into a COY Laboratories vinyl chamber, go through two automatic cycles to establish anaerobiosis to limit the amount of oxygen that is entering the chamber.
- 7. Plan ahead so all required materials are transferred into the chamber at one time to avoid multiple transfers.
- 8. Keep track of the oxygen concentration recorded by the anaerobic detector during experimentation. Even small levels can be absorbed into anaerobic buffers and interfere with kinetic studies. Extending the time between transfers will also limit oxygen contamination from building up as the catalysts will remove the contaminant before introducing more into the chamber.
- **9.** If stopped-flow experiments are planned for the next day with the same enzyme, the same GO used to scrub the stopped-flow spectrophotometer before can be used to scrub the system again.

4.11.Data analysis

The spectra collected from stopped-flow spectrophotometry can be analyzed to determine the observed rate (k_{obs}) under different reactant concentrations. The most basic studies of flavoenzyme chemistry observe the changes to the flavin at 450 nm to determine the observed rate constants during reduction and oxidation. Before analysis, traces collected at 700 nm, where no species absorb, can be subtracted from traces collected at 450 nm in order to compensate for baseline fluctuation. These data can then be plotted against time to show an exponential decay or rise. These data can be fitted to an exponential for decay (Eq. 3) and rise (Eq. 4) equations, where k_{obs1} is the observed rate constant of the fast reaction and k_{obs2} is the observed rate constant of the slow reaction, B₁ or B₂ are the amplitudes of the flavin change, and C is the final absorbance of the flavin during reduction (Eq. 3) or oxidation (Eq. 4).

$$A_{450} = B_1 e^{-k_{obs1}t} + B_2 e^{-k_{obs2}t} + C$$
(3)

$$A_{450} = B_1 \left(1 - e^{-k_{obs1}t} \right) + B_2 \left(1 - e^{-k_{obs2}t} \right) + C$$
(4)

5. OTHER USES FOR ANAEROBIC CHAMBERS

Beyond stopped-flow spectrophotometry, anaerobic chambers offer an opportunity to use different anaerobic techniques to study enzymes. These chambers offer a large anaerobic work space that, with a little creativity, can facilitate any type of experiment a researcher might be planning. An anaerobic chamber can be optimized to perform many of these applications. This section briefly describes some of other uses of an anaerobic chamber applied to enzymology.

5.1. Protein purification

One common application for an anaerobic chamber is its use in protein purification. Anaerobic protein purification is typically applied to metal containing enzymes where reactions with oxygen will inactivate the metal core (Echavarri-Erasun et al., 2014). An example setup involves placing an FPLC fraction collector and a sonicator inside of a chamber (Tsai & Tainer, 2018). Reducing agents can be added to the buffer and sample unless the enzyme or the column is sensitive to reduction.

Because anaerobic protein purification is commonly used to isolate metalloenzymes, multiple review papers discussing this method have been published (Echavarri-Erasun et al., 2014; Selmer, 2005; Tsai & Tainer, 2018).

5.2. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry is a common technique used to determine the binding of two molecules. The instrument measures the heat released or absorbed by the interaction of molecules. For the purpose of protein assays, ITC is regularly used to study the binding of substrates, inhibitors, peptides, and cofactors to a protein providing quantitative results within a few hours. With flavoenzymes in particular, it is sometimes also expected that specific ligands bind more effectively to the reduced form of the enzyme over the oxidized form. ITC experiments can be very useful in determining these changes in affinity; however, maintaining a reduced flavin is difficult without an anaerobic environment.

Most modern ITC instruments are small enough to comfortably fit inside an anaerobic chamber with the accompanying solution bottles for washing and waste collection. Using an ITC inside of a chamber allows anaerobic detection that is commonly used to determine interactions of reduced metals and enzymes (Kondapalli, Kok, Dancis, & Stemmler, 2008). During preparation, the enzyme sample and buffer stock are dialyzed aerobically and deoxygenated with high purity argon or degassed with only a vacuum (Henderson, Müller, Hausinger, & Emerson, 2015; Pop, Kolarik, & Ragsdale, 2004). Researchers have also added sodium dithionite or GO to the solutions to maintain anaerobiosis (Bou-Abdallah et al., 2002; Terpstra et al., 2014). With this setup, it is still possible to perform aerobic assays inside of the anaerobic chamber by using samples that have not been deoxygenated.

5.3. Ultraviolet-visible (UV-vis) spectrophotometry

UV-vis spectrophotometry offers other techniques for studying anaerobic enzyme reactions beyond that discussed with stopped-flow spectrophotometry. One of the simplest applications of UV-vis spectrophotometry using an anaerobic chamber is by placing the spectrophotometer inside of the chamber. This setup lends itself to easy preparation and detection of anaerobic enzyme assays (Lopez de Felipe & Hugenholtz, 2001; Rau & Stolz, 2003; Schulte et al., 1998). Most modern spectrophotometers are small enough to fit inside of a chamber leaving plenty of space for preparing enzyme assays. Setting up a spectrophotometer inside of a chamber will require a computer. Integrating the connections required for communications through the wire ports allows the computer to be outside of the chamber providing more space inside of the chamber to operate the instrument.

Another method that is used to perform anaerobic UV-vis spectrophotometry is using an anaerobic cuvette (Kantz, Chin, Nallamothu, Nguyen, & Gassner, 2005; Rothman, Helm, & Poulter, 2007; Zeghouf, Fontecave, Macherel, & Covès, 1998). This technique allows an investigator to study anaerobic reactions without the need of a spectrophotometer inside of a chamber. The solutions can be prepared inside of an anaerobic chamber and placed inside of an anaerobic cuvette with an airtight seal before being transferred out of the chamber for measurement on a UV-vis spectrophotometer. Anaerobic cuvettes can also be customized for specific assays, and some offer the ability to deoxygenate solutions directly inside of the cuvette using a Schlenk line.

Non-continuous assays can also be used to perform anaerobic experiments inside of a chamber. These assays can be used to obtain data quickly because multiple assays can be performed at once. Termination of the enzyme reaction is facilitated with either organic solvent, acid, or heat. This method is useful as oxygen deactivates the enzyme without precipitation and does not affect other factors in the assay. Discontinuous anaerobic assays can be used beyond UV–visible spectrophotometry, and have been used to prepare samples for high performance liquid chromatography (HPLC) studies (Gorny & Schink, 1994; Thiele et al., 2008).

6. SUMMARY AND CONCLUSIONS

In conclusion, the methods outlined in this chapter provide a practical approach to preparing and performing flavoenzyme assays studying the reductive and oxidative half-reactions with a stopped-flow spectrophotometer. There are no significant limitations from placing a stopped-flow spectrophotometer inside of an anaerobic chamber, and this setup decreases the amount of time spent deoxygenating solutions while also reducing the risk of oxygen contamination. Anaerobic chambers also offer a lot of space for other enzymology applications. The chamber cost and maintenance should be considered if a stopped-flow spectrophotometer is to be setup inside of an anaerobic chamber.

ACKNOWLEDGMENTS

Research reported in this publication was supported by grants from the National Institute of General Medical Sciences award R01GM094469, the National Science Foundation award CHE-1506206.

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Keywords: Stopped-flow spectrophotometry; Anaerobic chambers; Flavoenzymes; Reduction; Oxidation; Enzyme assays; Enzyme kinetics; Schlenk line; Anaerobiosis